UiT The Arctic University of Norway

Faculty of Health Sciences

Cardiovascular Research Group

Modulation of Cardiometabolic Health by Dietary Supplementation with Calanus oil

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List of papers

Paper I

Jansen KM, Moreno S, Garcia-Roves PM, Larsen TS. Dietary Calanus oil recovers metabolic flexibility and rescues postischemic cardiac function in obese female mice. Am J Physiol Heart Circ Physiol. 2019; 317(2):H290-H299. doi: 10.1152/ajpheart.00191.2019. Epub 2019 May 24. PMID: 31125256

Paper II

Kirsten M Jansen*, Synne S* Hansen, Kenneth B Larsen, Anne D Hafstad, Ragnar L Olsen, Terje S Larsen, Ellen Aasum. Hydrolysed wax ester from Calanus oil protects H9c2 cardiomyoblasts from palmitate – induced lipotoxicity (manuscript).

Paper III

Pauke C Schots*, Kirsten M Jansen*, Jakub Mrazek, Alice M Pedersen, Ragnar L Olsen, Terje S Larsen. Obesity-induced alterations in the gut microbiome in female mice fed a high-fat diet are antagonized by dietary supplementation with a novel, wax ester-rich, marine oil. Nutr Res 2020, 83:94-107. doi: 10.1016/j.nutres.2020.09.002. Epub 2020 Sep 9. PMID: 33049454

Review

Larsen TS, Jansen KM. Impact of Obesity-Related Inflammation on Cardiac Metabolism and Function. J Lipid Atheroscler **(review)**. 2021;10 (1): 8-23. doi: 10.12997/jla.2021.10.1.8. Epub 2020 Nov 10.

* Both authors contributed equally and shared first authorship

Abbreviations

ATP	Adenosine triphosphate
BMI	Body mass index
Cal	Calanus
CD36	Cluster of differentiation 36 (Fatty acid translocase)
DHA	Docosahexaenoic acid
DNP	2-4-Dinitrophenol
EPA	eicosapentaenoic acid
ER	Endoplasmic reticulum
FA	Fatty acid(s)
FABP	Fatty acid binding protein
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
GLUT4	Glucose transporter type 4
HFD	High fat diet
IKK	IκB kinase
IL	Interleukine
IRS	Insulin receptor substrate
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharides
MCP-1	Monocyte chemoattractant protein-1
NCD	Normal chow diet
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
Pal/Palm	Palmitate
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PUFA	Poly-unsaturated fatty acids
qPCR	quantitative polymerase chain reaction
SCFA	Short chain fatty acid
SDA	Stearidonic acid
SGLT-2	Sodium-glucose cotransporter-2
TAG	Triacylglycerol
TMA	Trimethylamine
TMAO	Trimethylamine-N-oxide
TNF-α	Tumor necrosis factor-alpha
UPR	Unfolded protein response
WE_H	Wax ester hydrolysate
WE	Wax ester

Summary of thesis

Obesity leads to alterations in myocardial metabolism, expressed as an increased utilization of fat for energy purposes at the expense of carbohydrate. This shift in energy metabolism is associated with impairment of ventricular function which can lead to heart failure. This thesis raises the question of whether dietary supplementation with Calanus oil, a novel marine oil derived from the marine copepod, Calanus finmarchicus (Norwegian, raudåte), can attenuate obesity-related changes in metabolism and associated co-morbidities.

The major finding in this thesis project was that dietary Calanus oil was able to prevent obesity-related shifts in myocardial metabolism and restore the capacity of the heart to utilize carbohydrate as energy source. In addition, we demonstrated that Calanus oil had a cardioprotective action, in the sense that recovery ventricular function following an ischemic insult of hearts from mice receiving high-fat diet with Calanus oil was superior to that of control hearts. To examine the mechanism involved, we exposed H9c2 cardiomyoblast to lipotoxic stress (palmitic acid) with and without micromolar concentrations of hydrolysed wax ester from Calanus oil (WE_H). Live cell imaging revealed major cell death in response to palmitiate-exposure, while co-incubation with WE_H almost completely rescued the cells. Further analyses suggested that WE_H protects H9c2 cardiomyocytes from lipotoxicity by attenuating the associated ER stress, as well as alleviating palmitate-induced impairment of autophagic flux. This thesis also showed that Calanus oil, at least to some extent, were able to antagonize obesity-induced changes in the gut microbiota composition. In conclusion, the present results show that dietary supplementation with Calanus oil has the potential to attenuate obesity-induced alterations in myocardial metabolism and, in addition, protect the heart towards ischemic stress, and may therefore be an alternative to anti-obesity/anti-diabetic drugs.

Chapter 1:

Introduction

1.1. Obesity and its comorbidities – a major health problem worldwide

Obesity is a metabolic disorder, which causes pathologic alterations in energy metabolism, leading to conditions such as type 2 diabetes and fatty liver disease which subsequent increased the risk of coronary heart disease and ischemic stroke (Finucane et al. 2011). Obesity is a growing health problem in both developed and developing countries, and in the last 20 -30 years the incidence of the disease has increased worldwide at an alarming rate. Thus, according to the World Health Organization, more than 1.9 billion adults (18 years and older) were overweight in 2016 (Organization 2016).

Previously, adipose tissue was thought to be an energy reservoir which can be mobilized during states of energy shortage (Hajer et al. 2008), but today we realize that adipose tissue, in addition to its role in energy homeostasis, is a major endocrine organ, secreting a large number of bioactive proteins and peptides, collectively referred to as adipokines (Trayhurn and Beattie 2001). The adipokine profile may vary between different adipose tissue depots and is significantly altered in obesity. Thus, in non-obese healthy adipose tissue, secretion of anti-inflammatory adipokines predominates, whereas secretion of pro-inflammatory adipokines dominates during obese states.

Adipose tissue expansion in obesity is often regarded as a consequence of an increased energy intake and/or a sedentary lifestyle, but in addition it is modified by both environmental and genetic factors (Barsh and Schwartz 2002; Zheng and Berthoud 2008). Subcutaneous adipose tissue, probably the largest fat depot in the body, expands through recruitment and differentiation of adipose precursor cells, but when its storage capacity is exceeded, excess energy intake leads to fat accumulation in alternative locations, in particular intra-abdominal depots and ectopic tissues such as the liver, skeletal muscle and heart, where accumulated lipids create a condition of lipotoxicity (see figure below). The expansion of adipose tissue is reflected in hypertrophied adipocytes, which implies that the distance between the blood-bearing vessels increases and oxygen diffusion becomes insufficient (Cinti et al. 2005). Characteristically, the adipose tissue of obese individuals shows lower

blood flow, higher vasoconstriction, and lower capillary density than adipose tissue in non-obese individuals (Schenk et al. 2008). The consequent development of local hypoxia is believed to trigger both local and systemic release of inflammatory adipokines from the enlarged adipocytes.

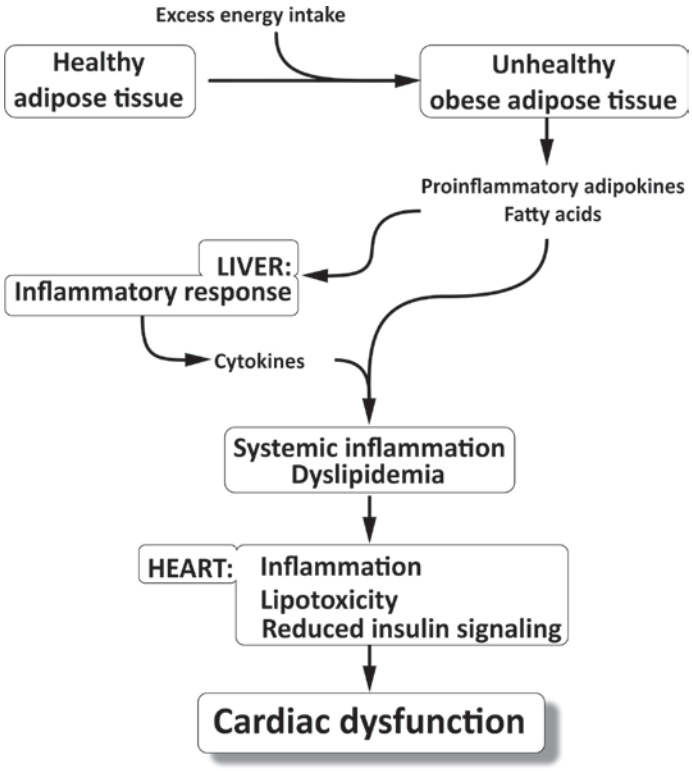

Figure showing that increasing visceral obesity causes inflammatory responses and metabolic dysregulation in fat and liver tissue. This condition involves infiltration of monocytes and macrophages and subsequent secretion of proinflammatory adipokines and elevated release of free fatty acids, leading to systemic inflammation, which promotes insulin resistance in several organs, including the heart. In addition, an elevated supply of lipids (free and esterified fatty acids) exceeds the fatty acid oxidation capacity and causes lipotoxicity in the myocardium, eventually leading to cardiac dysfunction (figure borrowed from the review).

1.2. Altered adipose tissue metabolism and increased production of inflammatory adipokines in obesity

Excess energy intake over time represents an increased demand for lipid storage beyond the normal capacity of the adipocytes. Sun et al. (Sun et al. 2013) documented increased interstitial fibrosis with abnormal collagen deposition in white adipose tissue during the development of obesity, which may reduce extracellular matrix flexibility and decrease the tissue plasticity.

Along with these alterations in adipose tissue structure, major changes in the secretory output occur, characterized by increased production of pro-inflammatory adipokines; tumor necrosis factor-α (TNF-α), interleukin (IL) -6, and IL-1β as well as monocyte chemoattractant protein-1 (MCP-1) (Kim and Lee 2014; Panee 2012). On the molecular level, this process includes activation of the c-Jun N-terminal kinase (JNK), as well as IκB kinase (IKK) β and nuclear factor kappa light chain enhancer of activated B cells (NF-κB) (Solinas and Karin 2010), which in turn regulate protein phosphorylation and cellular transcriptional events (Marcus et al. 2013). MCP-1 induces recruitment and infiltration of new macrophages(Sun et al. 2011), and resident macrophages in adipose tissue express a switch from an anti-inflammatory (M2) state to a pro-inflammatory (M1) state (Chawla et al. 2011; Skurk et al. 2007), thereby exacerbating the obesity-associated inflamed status of the tissue (Weisberg et al. 2003; Wood et al. 2009; Xu et al. 2003) and creating a so-called low-grade inflammatory state. More than 90% of M1-type macrophages are localized to dead adipocytes, forming "crown-like structures", which are characteristic components of the immuno-histological picture of adipose tissue in both obese mice and humans (Cinti et al. 2005). In addition, release of inflammatory molecules from visceral fat to the liver leads to hepatic inflammation, due to direct drainage into the portal vein (Choe et al. 2016). In this way, inflamed adipose tissue and liver collectively create a systemic inflammation, which in turn can negatively impacts on metabolic processes in vital organs like liver, skeletal muscle and heart.

1.3. The adipo-cardiometabolic axis

Obesity is associated with risk factors for cardiovascular disease, such as diabetes, high cholesterol, high blood pressure and metabolic syndrome (Litwin 2008; Zhang et al. 2014), but obesity also represents an independent major risk factor for the development of cardiovascular diseases (Cercato and Fonseca 2019). Cardiovascular disease is not a specific theme for this thesis, but it should be stressed that release of adipokines from enlarged adipose tissue plays a key role in the interorgan cross talk, not only between adipose tissue and the cardiac vasculature (referred to as the adipo-cardiovascular axis) (Taube et al. 2012), but also between adipose tissue and myocardial metabolism, which could be coined the *adipo-cardiometabolic axis*. Chronic low-grade inflammation and increased adipose tissue lipolysis lead to altered metabolism in the heart. Under normal physiological conditions, insulin inhibits lipolysis in adipose tissue and accelerates triacylglycerol (TAG) synthesis, but in obesity altered protein phosphorylation and cellular transcriptional events lead to insulin resistance, which accelerates TAG mobilization and lead to elevated circulating levels of fatty acids (FA) and TAG (Zhang et al. 2011) and increased myocardial FA uptake and oxidation (Lopaschuk et al. 2010; Rimm et al. 2018; Ljubkovic et al. 2019; Liu et al. 2014; Ussher et al. 2012). In the normal heart approximately 50%–70% of the energy (Adenosine triphosphate, ATP) requirement is produced by oxidation of long-chain FAs (bound to albumin or esterified in circulating triglycerides). Oxidation of carbohydrates, lactate and, to some extent, also ketone bodies and amino acids cover the rest. In the 1960s, Sir Philip Randle performed landmark studies showing how metabolic products of increased FA oxidation can inhibit glucose uptake in muscle (Randle et al. 1963). This mechanism, subsequently known as the Randle cycle, is the basis of metabolic flexibility in healthy individuals, which allows energy-requiring organs such as heart and skeletal muscle to switch between fuels depending on the nutritional and hormonal status. Thus, in obesity and insulin-resistant states when the supply of free and esterified fatty acids is increased, myocardial energy production becomes heavily dependent on fatty acid oxidation, covering as much as 90% of the energy requirement (Lopaschuk et al. 2007).

The substrate transporters glucose transporter type 4 (GLUT4, for glucose) and CD36 (fatty acid translocase) for FAs, play a central role in this dynamic balance of substrate utilization (Chanda et al. 2016). CD36 plays a central role in facilitating cellular long-chain FA uptake across the plasma membrane, acting in concert with other membrane proteins, such as FA-binding protein (Glatz and Luiken 2018), but in obesity translocation of CD36 to the sarcolemma is increased, which markedly enhances the uptake and oxidation of long-chain FA. In obese Zucker rats, persistent relocation of CD36 to the sarcolemmal membrane could partially account for the increased fatty acid uptake and oxidation (Coort, Luiken, et al. 2004). Both CD36 and fatty acid binding protein (FABP) expression are increased also in hearts from diabetic animals (Coburn et al. 2000; Coort, Hasselbaink, et al. 2004), explaining in part the elevated fatty acid uptake and development of metabolic dysregulation in the setting of diabetes.

Not all FAs entering the cardiomyocytes, however, are utilized for oxidative purposes, and long-chain FAs in the form of acyl-CoA provide substrates for nonoxidative processes such as triglyceride, diacylglycerol, and ceramide synthesis (Unger 2002; Chess and Stanley 2008). Accumulation of these substances can impair insulin signaling by activating a similar set of kinases as already mentioned above as part of the inflammatory response in obese adipose tissue (JNK, IKK-β, NF-κB and PKC). This causes tyrosine phosphorylation of insulin receptor substrate (IRS)-1, followed by reduced Phosphoinositide 3-kinase (PI3K) and Akt activity, resulting in reduced GLUT4 translocation to the sarcolemma and, finally, reduced glucose uptake. This development of insulin resistance significantly reduces the metabolic flexibility of the heart (as well as skeletal muscle) (Rijzewijk et al. 2009), so that myocardial energy production becomes primarily dependent on FA oxidation. As a consequence, accumulation of the intermediates of FA metabolism in cardiomyocytes results in a state of lipotoxicity (Unger 2002; Paulus and Tschöpe 2013).

1.4. The obesity paradox

Although the adverse health consequences of obesity in the general population are well documented, it may be protective and associated with greater survival in certain groups of people, such as elderly individuals, or those with certain chronic diseases (Flegal et al. 2013; Carnethon et al. 2012). In particular, studies of patients with heart failure have revealed an inverse correlation between body mass index (BMI) and heart failure outcome (mortality), which is the main argument for the existence of an "obesity paradox". Thus, heart failure patients with a BMI between 30.0 and 34.9 had lower mortality than those considered non-obese (Habbu et al. 2006). Other studies have questioned the validity of the obesity paradox, and argued that BMI measurements do not give information of the amount of lean body mass or body fat (Habbu et al. 2006; Preston and Stokes 2014). Therefore, the "improved health" of obese heart failure patients might not necessarily be linked to increased fat mass, but more to the protective effect of increased lean body mass (McAuley and Blair 2011; Carbone et al. 2017). Also, obese patients could be healthier than their lean counterparts, due to more aggressive treatment following disease onset (Diercks et al. 2006). Another, and probably the main reason for the inverse correlation between BMI and heart failure outcome could be that underweight patients have poor outcome due to the deleterious effects of weight loss (cachexia). Classifying these individuals as lean implies an increase in the mortality rate in the normal and underweight categories of BMI, while lowering the risk in the higher BMI categories (McAuley and Blair 2011). Similarly, smokers tend to be leaner, and are also subject to higher mortality rates. Therefore, adjustment for smoking greatly reduced the mortality estimates in the underweight group, as well as strengthening the estimates in the overweight and obese groups (Stokes and Preston 2015). At any rate, it should not be neglected that people with greater degrees of obesity have increased risk of events (Romero-Corral et al. 2006; Oreopoulos et al. 2008), and circumstantial evidence suggests that a "U-shaped" outcome curve according to BMI for patients with heart failure may actually exist, in which mortality is greatest in cachectic patients; lower in

normal, overweight, and mildly obese patients; but higher again in more severely obese patients (Habbu et al. 2006).

1.5. Palmitate-induced lipotoxicity

Liportoxicity is defined as cellular toxicity in the presence of an abnormal accumulation of fat in non-fat tissues. Palmitic acid is a common fatty acid in the circulation, and elevated level of this fatty acid can lead to a lipotoxic state, which is associated with endoplasmatic reticulum (ER) stress, apoptosis and dysfunctional autophagy in cardiomyocytes.

The ER is a subcellular organelle producing proteins needed for the rest of the cell to function (Griffiths et al. 1984). The smooth part of ER has an important role to ensure the proper folding and maturation of newly secreted proteins and transmembrane proteins. Thus, numerous molecular chaperones in the ER lumen bind to the proteins and prevent them from aggregation (Uppala et al. 2017; Hartl et al. 2011). For this reason, ER is regarded a quality checkpoint which allows the existence of only properly folded proteins (Hwang and Qi 2018).

Increasing amounts of lipid accumulation, in the form of palmitate overload, leads to a loss of ER homeostasis (ER stress). In order to resolve ER stress, cells activate a homeostatic intracellular signaling network cumulatively called the unfolded protein response (UPR) (Hartl et al. 2011). UPR recuperates ER function by attenuating protein synthesis or by inducing the expression of various genes encoding for molecular chaperones and enzymes that promote protein folding and post-translational modification. If ER protein homeostasis is not restored, the prolonged activation of the UPR may initiate apoptotic cell death via the up-regulation of the C/EBP homologous protein (CHOP).

In addition, autophagy, the intracellular lysosome-mediated degradation pathway for recycling and eliminating damaged proteins and organelles, has also emerged as an essential protective mechanism during palmitate induced lipotoxicity (Park et al. 2015). Although, nutrient-induced

changes in autophagy seems to vary between cell types (Jiang et al. 2017; Miyagawa et al. 2016; Portovedo et al. 2015; Park et al. 2015), palmitate has been reported to impaired autophagic flux H9c2 cardiomyoblast (Jaishy et al. 2015). Therefore, several findings suggest that increased autophagy has a protective effect against cell death during cellular stress, while sustained activation may have detrimental effects (Shintani and Klionsky 2004; Park et al. 2015).

1.6. The gut microbiome and metabolic disorders

The role of gut microbiota in human health has received significant interest in recent years, and emerging evidence suggests that microbes resident in the human intestine represent a key environmental factor contributing to obesity and metabolic disorders, such as insulin resistance and type 2 diabetes mellitus (Huttenhower et al. 2012; Power et al. 2014; Flint et al. 2017; Flint et al. 2012; Bäckhed et al. 2004; Ley et al. 2005). Although the molecular link between the gut microbiota and these conditions is poorly understood, it is clear that altered gut microbiota profiles can lead to changes in gut permeability, initiating a chronic low-grade inflammatory state (Honda and Littman 2016; Sanz and De Palma 2009). As a consequence, endotoxins such as lipopolysaccharides (LPS) can pass through the damaged intestinal wall (Ghoshal et al. 2009) and lead to a systemic inflammation, which is considered important for the pathogenesis of the inflammatory-induced metabolic disorders mentioned above (Power et al. 2014; Hildebrandt et al. 2009). Of particular interest is the identification of a new gut microbiota-initiated pathway by which gut microbes produce the metabolite trimethylamine (TMA) by digestion of nutrients common in Western diets (phosphatidylcholine, choline, and L-carnitine) (Wang et al. 2011; Koeth et al. 2013; Gregory et al. 2015; Romano et al. 2015). Absorbed TMA is subsequently oxidized to trimethylamine-N-oxide (TMAO) by hepatic flavin-containing monooxygenases in the liver of the host (Chalmers et al. 2006; Wang et al. 2011). Importantly, several studies have reported that increased plasma levels of TMAO are associated with an increased risk of adverse cardiovascular events in humans (Wang et al. 2011;

Koeth et al. 2013; Tang et al. 2013; Suzuki et al. 2016). In diabetics, elevated circulating TMAO levels are associated with poor outcomes after cardiovascular disease (Lever et al. 2014; Dambrova et al. 2016).

It is also well accepted that diet has a strong influence on the microbial communities within the gastrointestinal tract of animals (Hildebrandt et al. 2009; Ravussin et al. 2012; Hamilton et al. 2015). Thus, high-fat feeding in mice leads to an imbalance in the gut microbial community (dysbiosis) with adverse effects on intestinal health, including abundance of LPS-producing bacteria, impaired intestinal barrier function (Moreira et al. 2012; Ji et al. 2011; Cani and Delzenne 2011) and subsequent diffusion of LPS to the bloodstream (Laugerette et al. 2011). Animal studies have traditionally focused on the relationship between the two major bacterial phyla *Bacteroidetes* and *Firmicutes*, where an increase in the *Firmicutes/Bacteroidetes* ratio has been linked to weight gain and other metabolic conditions. Moreover, low *Firmicutes/Bacteroidetes* ratio due to intake of omega-3 poly-unsaturated fatty acids (PUFA) (Liu et al. 2012; Yu et al. 2014) is associated with reductions in body weight gain and white adipose tissue inflammation (Caesar et al. 2015), as well as reduced adiposity index. It has been suggested that dietary PUFA affect the attachment sites for the gastrointestinal microbiota, possibly by modifying the fatty acid composition of the intestinal wall (Kankaanpää et al. 2001). The impact of marine PUFA on the gut microbiota in humans is relatively sparse, and early studies by Rajkumar et al. (Rajkumar et al. 2014) failed to detect any major effects on the gut microbiota composition. Later studies on both healthy and obese individuals showed that omega-3 PUFA supplements were able to reverse this condition by restoring the *Firmicutes/Bacteroidetes* ratio, and increasing *Lachnospiraceae* taxa (Balfegó et al. 2016; Noriega et al. 2016; Pu et al. 2016; Watson et al. 2018), both associated with an increased production of the anti-inflammatory short-chain fatty acid (SCFA) butyrate (Pu et al. 2016; Watson et al. 2018; Costantini et al. 2017). Moreover, in animal studies omega-3 PUFA attenuated endotoxemia by increasing LPS-suppressing bacteria, *Bifidobacteria*, and decreasing LPS-producing bacteria,

Enterobacteria (Kaliannan et al. 2015). Taken together, these results have led to the view that omega-3 PUFAs can be considered as prebiotics, able to restore gut eubiosis in some pathological conditions**.**

1.7. Treatment strategies

Obesity, in particular visceral obesity is a risk factor for coronary artery disease, which results from cholesterol plaque buildup in the arteries of the heart. Other heart problems related to obesity are heart failure, atrial fibrillation and sudden cardiac death. These health complications affect quality of life, reduce average life expectancy and place an enormous burden on health care resources. This situation calls for effective strategies to mitigate the obesity pandemic, and in order to be successful one has to acknowledge that obesity is a heterogeneous, chronic and complex disease. Thus, obesity is more than a question of "calories in and calories out", and reduced calorie intake and increased calorie expenditure via daily exercise alone have therefore not been effective in achieving lasting weight loss. Therefore, treatment beyond the first line interventions of lifestyle is required for achieving lasting weight loss.

The pharmaceutical industry has developed a number of anti-obesogenic medications, which work through mechanisms that either increase energy expenditure, reduce energy intake and/or reduce energy absorption. However, several of these agents have been withdrawn from the market due to safety concerns (Van Gaal and Dirinck 2016). This includes 2-4-Dinitrophenol (DNP) was one of the first anti-obesity drugs used in the 1930s. It was shown to cause weight loss by uncoupling oxidative phosphorylation, thereby leading to an elevated metabolic rate and increased fat consumption. However, it was soon realized that there were serious side effects by the use of DNP (e.g. cataract), in some cases even deaths (Colman 2007). It was therefore withdrawn from the marked in 1938.

Sodium-glucose cotransporter-2 inhibitors (SGLT-2 inhibitors), are novel drugs which promote weight loss via reduced energy absorption (Van Gaal and Dirinck 2016; Derosa et al. 2012; Torgerson et al. 2004). The main effect of the relatively newly developed SGLT-2 inhibitors (e.g. empagliflozin, dapagliflozin and canagliflozin) is to inhibit glucose reabsorption in the renal proximal tubular cells and thereby reduce blood glucose levels through increased glycosuria. These drugs were initially developed as anti-diabetic drugs, but they have also been shown to reduce body weight through reductions in (visceral and subcutaneous) fat mass, and there is hope that they could contribute significantly in the combat against obesity. Another class of anti-diabetic drugs with weight-reducing potential that has become available in recent years is the glucagon-like peptide-1 (GLP-1) receptor agonists. These drugs are so-called incretin mimetics, which means that they act like incretin hormones such as glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide (GIP). They bind to GLP-1 receptors and stimulate glucose-dependent insulin release from the β-cells in the pancreas to avoid hyperglycemia. The beneficial effect of incretin mimetics on weight loss is associated with suppression of appetite and food intake via central mechanisms (Näslund et al. 1999). They also reduce food intake (and the feeling of hunger) by slowing gastric emptying. Exenatide, the first in this new class of drugs (which is also used in the present PhD project), was introduced to the market in 2005 (USA) and 2007 (Europe) under the trade name Byetta, while Liraglutide became available a couple of years later. Exenatide shares 53% homology with native GLP-1 and has a half-life of about 2 hours, while liraglutide has 97% sequence homology to native GLP-1 and a half-life following subcutaneous administration of approximately 13 hours (Eng et al. 1992).

A further discussion regarding the pharmacology, efficacy, safety, and tolerability of the various anti-obesity medications is beyond the focus of this thesis. However, since the thesis deals with the effect of marine PUFA preparations as anti-obesogenic and anti-diabetic treatments, we will describe here the source and chemical composition of Calanus oil, as well as briefly mention previous results

obtained with this particular oil. The use of marine omega-3 FA preparations for health promotion purposes will be discussed further in the “Results and Discussion” section.

1.8. Calanus oil

Calanus oil is extracted from the marine copepod Calanus *finmarchicus*, the most abundant crustacean and one of the dominating food sources for fish in the North Atlantic (Skjoldal and Saetre 2004). It is only 3-4 mm long, but still provides approximately 50% of annual biomass production in the North Atlantic (Lalli and Parsons 1997). Calanus *finmarchicus* feed on various forms of phytoplankton, and during the spring and summer it stores large amounts of energy in the form of oil, which can account for as much as 50% of its dry weight. It is therefore an important prey item for many fish species, such as herring and mackerel (Prokopchuk and Sentyabov 2006).

Oil extracted from Calanus *finmarchicus* has a unique chemical composition (Table 1). The content of eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) is relatively low compared to other marine oils, but it has a high content of stearidonic acid (SDA, 18:4, n-3), as well as other long chain mono-unsaturated fatty acids, such as gondoic acid (20:1 n-9) and cetolieic acid (22:1 n-11) (Bergvik et al. 2012). Furthermore, the fatty acids in Calanus oil is mostly bound as monoesters (wax esters), where the fatty acids are linked to monounsaturated long-chain fatty alcohols, eicosenol (20:1n-9) and docosenol (22:1n-11) (Pedersen, Salma, et al. 2014; Pedersen, Vang, et al. 2014). In krill- and fish oil the larger part of the fatty acids is bound in phospholipids and triglycerides, respectively. Calanus oil also contains proteins, vitamins, minerals, phytosterols, as well as a high amount of the antioxidant astaxanthin (Naguib 2000), the latter giving the oil its characteristic red color.

Table 1. Fatty acid content (mg/g lipid) of Calanus Oil and the experimental diets used in this thesis. Modified from Pedersen et al. (Pedersen, Salma, et al. 2014).

Fatty acids	Calanus Oil	HFD	HFD + Cal
14:0	64.42	10.41	11.95
16:0	45.05	173.65	149.83
18:0	2.42	106.63	92.16
20:0	0.40	1.70	1.37
16:1n-7	17.17	10.95	9.98
18:1n-7	1.53	15.84	13.52
18:1n-9	15.54	243.82	208.42
20:1n-9	24.01	4.74	5.64
20:1n-11	3.90	nd	nd
22:1n-9	2.63	nd	nd
22:1n-11	43.33	nd	2.20
24:1n-9	2.81	nd	nd
18:2n-6	6.64	133.04	116.06
18:3n-3	13.72	12.49	11.67
18:4n-3	69.58	nd	4.54
20:2n-6	0.71	3.15	2.69
20:4n-6	1.39	0.48	1.15
20:5n-3	54.73	nd	3.35
22:5n-3	2.96	nd	nd
22:6n-3	39.35	nd	2.81

nd, not determined.

Digestion and utilization of wax esters is poorly understood (Gurr et al. 2016), although it is evident that humans are able to hydrolyze waxes and absorb the liberated fatty acids and alcohols (Hargrove et al. 2004). Furthermore, previous studies in mice have shown that the wax esters are hydrolyzed and absorbed in the intestine, and that the wax ester-derived fatty acids are incorporated in liver and white adipose tissue (Pedersen, Salma, et al. 2014). Dietary supplementation with Calanus oil leads to a significant increase in the omega-3 index (increase in relative content of omega-3 fatty acids in red blood cell membranes) (Wasserfurth et al. 2020). Dietary lipids, such as triacylglycerol and phospholipids are quickly digested and absorbed in the upper parts of the gastrointestinal tract and will normally not reach the lower part (Carey et al. 1983). Wax esters are hydrolyzed by a bile salt-dependent pancreatic carboxyl esterase (Hargrove et al. 2004). They are relatively hydrophobic and therefore more difficult to emulsify, and as result wax esters may exhibit

a longer retention time (Cowey and Sargent 1977; Verschuren and Nugteren 1989), allowing release of the liberated fatty acid and fatty alcohols in the distal part of the intestine.

Finally, several studies from the Cardiovascular Research Group at the Department of Medical Physiology have documented that dietary supplementation with small amounts of Calanus oil during high-fat feeding mitigates intra-abdominal fat deposition and at the same time blunts the obesity-induced inflammation locally in the tissue (Höper et al. 2013; Höper et al. 2014). Other beneficial health effects of the oil include prevention of angiotensin II-induced hypertension and reduced cachexia during angiotensin II stress (Salma et al. 2016).

Chapter 2:

Aims of the Thesis

Aims of the thesis

Paper I: Obesity induced by high-fat diet is known to induce alterations in myocardial metabolism and impaired tolerance to ischemia-reperfusion. The aim of this study was to examine if dietary supplementation with Calanus oil could prevent obesity-induced alterations in myocardial metabolism and, additionally, whether this could protect the heart against ischemic injury.

Paper II: Paper I revealed that hearts from mice given high-fat diet supplemented with Calanus oil showed an improved functional recovering following ischemia-reperfusion. Since we were unable to use animals, we switched to cell studies, using H9c2 embryonic rat cardiomyoblast which were stressed by exposure to palmitic acid. In this paper, our aim was to explore potential mechanisms which could explain the cardio-protective effects of Calanus oil.

Paper III: Obesity is characterized by unfavorable alterations in the composition and function of the gut microbiome. The aim of this study, we took advantage of the anti-obesity action of Calanus oil and tested whether dietary intake of the oil also could prevent or antagonize obesity-induced alterations of the gut microbiome.

The review: This is an invited review, focusing on the role of obese adipose tissue in the development of metabolic disturbances, and their consequences for metabolic and functional derangements in the heart. We elaborate on how expansion of adipocytes during the development of obesity gives rise to an unhealthy adipose tissue, characterized by a so-called low-grade inflammation.

Chapter 3:

Methodological considerations

2. Methodological consideration

2.1. Animals and dietary regimen

Diet-induced obese C57Bl/6J mice were used as experimental model in this thesis. This mouse strain is well characterized and reported to be highly susceptible to diet-induced obesity and diabetes. It is therefore widely used for studying the metabolic and physiological alterations that occur under these conditions. In order to induce obesity we fed the mice with a high-fat diet containing 45% energy from fat (TestDiet 58V8), while the diet of the lean controls contained only 10% energy from fat (TestDiet 58Y2). High-fat diet supplemented with Calanus oil was prepared by TestDiet (IPS, Notts, UK) by replacing 2% (w/w) of lard with 2% Calanus oil. Mice on HFD were also treated with the glucagon-like peptide-1 (GLP-1) receptor agonist, exenatide, which was administered via mini-osmotic pumps (Alzet Micro-Osmotic Pump Model 1004, Cupertino, CA, US). Based on previous experience (Höper et al. 2014), we subjected the mice to a 12 weeks fattening period, which was followed by 8 weeks treatment with either Calanus oil or exenatide. The experiments were approved by the local authority of the National Animal Research Authority in Norway. They were housed at 23°C (3 animals per cage) under a reversed light-dark cycle (12-h:12-h dark-light) with ad libitum access to food and drinking water.

2.2. *Ex vivo* heart perfusion

Ex vivo heart perfusion has turned out to be an important tool for characterizing cardiac function of small rodents. It is important to remember that the heart has been removed from its *in vivo* natural milieu. The advantage of this methodology is that it provides easy control of substrate and loading conditions. In addition, the heart is not influenced by any hormonal or neural regulations, allowing high level of reproducibility and comparability of the acquired data. There are two main models of perfusion; (1) the working heart perfusion where the heart is filled via the left atrium (from a pre-load chamber) and the left ventricle ejects its content against an external pressure (afterload)

and (2) the retrogradely perfused Langendorff mode, in which the left ventricle exerts pressure against an intraventricular fluid-filled balloon. Although heart perfusion in the working mode offers excellent possibilities to study cardiac physiology (e.g. by altering pre- and afterload settings) and performance (cardiac output, pressure-volume analysis), it is technically more demanding, since it requires cannulation of both aorta and the left atrium. More importantly, recovery of post-ischemic function is often insufficient to lift the afterload column, meaning that the risk for not obtaining proper post-ischemic functional data is high. Although the non-working Langendorff heart does not produce external work, it will generate pressure changes in the intraventricular balloon which can be recorded by a pressure transducer. Therefore, we used the Langendorff mode to record the contractile properties of the heart during normal control conditions, as well as after a 20 min. no-flow ischemic period (paper I of the present thesis).

2.3. Myocardial substrate utilization

Rates of myocardial fatty acid and glucose oxidation were measured by radioisotope technique when the hearts were stabilized in the pre-ischemic period, using ^{3}H-labelled palmitate and ^{14}C-labelled glucose, respectively. We used a recirculating airtight perfusion system, which allowed timed collections of perfusate for measuring $^{14}CO_2$ released from oxidation of ^{14}C-labelled glucose, as well as $^{3}H_2O$ from oxidation of ^{3}H-labelled palmitate previously (Belke et al. 1999; Midwood and Univ 1990; Aasum et al. 2003). Samples of the perfusate were collected in sealed tubes equipped with a filter paper with hyamine hydroxide, and $^{14}CO_2$ bound as bicarbonate was released and trapped on the filter paper by acidification of the samples. Gaseous $^{14}CO_2$ from the oxygenator was trapped by bubbling it through hyamine hydroxide (Barr and Lopaschuk 2000). $^{3}H_2O$ from oxidation of palmitate was measured using was recovered by vacuum sublimation (Midwood and Univ 1990). The beauty with this methodology is that it allows simultaneous measurement of utilization of the two major substrates in the same heart, and in this way detects any disturbances in the normal balance

between fat and carbohydrate consumption in response to metabolic diseases. A limitation, however, is that oxidation of alternative substrates such as lactate, ketone bodies and protein is not taken into account.

2.4. H9c2 cardiomyoblasts

In April 2017 the animal facility at the Health Sciences Faculty closed down due to expansion/renovation of the unit. The plan was to reopen the unit in the spring of 2018 but, unfortunately, the constructors faced technical difficulties, and reopening was heavily delayed. Repeated promises of reopening did not come true, and therefore, we decided to switch experimental model from mice to a cardiac cell line – so-called H9c2 cells. These cells are derived from embryonic rat hearts (cardiac myoblasts) and regarded as an alternative for cardiomyocytes in order to study pathophysiological mechanism. However, the cells do not show contractile activity and their energy consumption is primarily to maintain ionic homeostasis (Watkins et al. 2011). Extrapolation of results obtained with these cells to a beating heart should therefore be done with caution. The cells were cultured and stimulated in culture medium, consisting of high glucose Dulbecco's modified eagles medium (DMEM D5796, Sigma-Aldrich, St. Louis MO, USA) containing 10% fetal bovine serum (FBS, Sigma-Aldrich), 100 U/mL penicillin and 100 µg/mL streptomycin (p0781, Sigma-Aldrich). The cells were incubated in 5% CO_2 at 37°C.

In order to mimic the metabolic stress of obesity, we exposed H9c2 cells to a toxic concentration of palmitic acid. Cell viability was examined by live cell imaging, using a Zeiss Cell discoverer (Carl Zeiss Microscopy GmbH 07745 Jena, Germany). This provides a unique combination of a stable box, darkroom and integrated inverted research microscope which allows observation of live samples over a number of days and imaging several multiwell plates under optimal controlled incubation conditions, such as light, temperature and CO_2. The results obtained by the Zeiss Cell discoverer were confirmed by xCELLigence real-time cell analysis (xCELLigence Real Time Cell Analyzer, Agilent

Technologies, Santa Clara, CA 95051, USA). This instrument uses noninvasive electrical impedance monitoring to quantify cell proliferation and morphology in a real–time manner.

2.5. Separation and hydrolysis of wax ester from Calanus oil

In order to make the omega-3 fatty acids in the Calanus oil accessible to the H9c2 cells, we first isolated the neutral lipids of the oil containing the wax ester, as described by Vang et al. (Vang et al. 2013). The wax ester was subsequently separated and hydrolysed into its fatty acid and fatty alcohol constituents. Thin layer chromatography was used to confirm complete hydrolysis of the purified wax ester.

2.6. Fatty acid analysis

Fatty acids isolated from red blood cell membranes, colon wall and adipose tissue were methylated by dissolving the respective samples in 2 mol/L HCl in methanol with 0.05% BHT and heated for 2 hours at 100 °C. Afterwards, the fatty acid composition was determined by gas chromatography using an Agilent 6890N (Agilent Technologies, Santa Clara, CA, USA) gas chromatograph with a Varian CP7419 capillary column (Varian Inc, Palo Alto, CA, USA). The content of the individual fatty acids in the samples were expressed in percent of the total fatty acid content.

2.7. Next generation sequencing

For the third study we analyzed microbiota in fecal samples from the mice. This was not our field of expertise, so we asked help from collaborators in Prague. They performed these experiments in short: Bacterial DNA was isolated using a standardized kit. To continue 16s rDNA amplification as performed to prepare PCR amplicons of the V4-V5 region of 16S rRNA according to Fliegerova et al. (Fliegerova et al. 2014).

Chapter 4:

Summary of results

Paper I: Dietary Calanus oil recovers metabolic flexibility and rescues postischemic cardiac function in obese female mice

In this study, female mice were fed a high-fat diet (HFD, 45% energy from fat) for an initial 12 week period, and in the following 8 weeks they either continued on the same diet, received HFD supplemented with 2% Calanus oil, or HFD combined with exenatide infusion (mini-osmotic pumps). A fourth group, receiving normal chow (NCD, 10% energy from fat) throughout the whole 20 week period, served as lean controls. Both the Calanus oil and exenatide group showed reduced adiposity index after the treatment, relative to the HFD group.

Glucose oxidation was significantly impaired, while fatty acid oxidation was increased in hearts from HFD mice. Both dietary supplementation with Calanus oil and exenatide infusion, however, restored the capacity of the heart to oxidize glucose. Somewhat surprisingly, measurements of ventricular function following ischemia-reperfusion showed better recovery of HFD hearts vs. hearts from lean controls. More importantly, however, postischemic functional recovery of hearts from mice who had received HFD supplemented with Calanus oil was superior to that of mice given non-supplemented HFD. Post-ischemic ventricular function of hearts from HFD mice treated with exenatide was not different from that of lean controls.

In summary, dietary supplementation with Calanus oil or administration of exenatide counteracted obesity-induced derangements of myocardial metabolism by restoring the myocardial capacity for glucose oxidation, while at the same time attenuating the dependency of fatty acid oxidation. Dietary Calanus oil supplementation also protected the heart from ischemia, which could have implications for the prevention of obesity-related cardiac disease.

Paper II: Hydrolysed wax ester from Calanus oil protects H9c2 cardiomyoblast from palmitate – induced lipotoxicity

In this study, we focused on possible mechanisms by which Calanus oil could afford cardioprotection, as demonstrated in paper I. Rat neonatal cardiomyblast (H9c2 cardiomyoblast) were exposed to lipotoxic stress in the form of high concentrations (100 μM) of palmitic acid with and without micromolar concentrations of hydrolysed wax ester from Calanus oil (WE_H), containing the liberated fatty acids and fatty alcohols.

Exposure to palmitate caused a major reduction in cell viability, so that around 80% of the cells were dead after 20 h incubation. In contrast, co-incubation with WE_H resulted in a dose-dependent increase in cell survival, where 10 μM WE_H almost completely prevented cell death. ER stress and autophagy are cellular pathways involved in palmitate-induced lipotoxicity, and we were able to show that ER stress was greatly reduced by co-incubation with WE_H, as shown by a significantly decrease in CHOP expression and a reduction in CHOP nuclear translocation. Furthermore, palmitate-induced LC3B vesicles and p62 inclusion bodies were abolished after co-incubation with WE_H. Thus, these data suggest that hydrolysed wax ester from Calanus oil protects H9c2 cardiomyoblast from lipotoxic stress by attenuating the associated ER stress, as well as alleviating palmitate-induced impairment of autophagic flux.

Paper III: Obesity-induced alterations in the gut microbiome in female mice fed a high-fat diet are antagonized by dietary supplementation with a novel, wax ester–rich, marine oil

Obesity is known to alter the composition and function of the gut microbiota. In this paper, we examined the effect of high-fat diet on the gut microbiota composition, and specifically whether Calanus oil could prevent such alterations.

To test this hypothesis, we utilized fecal samples from the colon of a random selection of mice from those used in paper I. Thus, samples were taken from mice which were initially subjected to a 12 week period on high-fat diet (HFD) and thereafter given HFD with or without 2% Calanus oil for the following 8 weeks. For comparative reasons, we also included a group of HFD mice that was treated with the anti-diabetic compound, exenatide, during the 8 last weeks of the feeding period. Mice fed on ordinary chow throughout the whole 20 week period served as lean controls.

Bacterial DNA was isolated from feces samples, followed by 16S rDNA amplification and next-generation sequencing. The sequences were subsequently used for taxonomical identification and functional analyses. Average species diversity and differences between the diet groups were expressed as α and β diversity, respectively.

HFD altered the gut microbiota composition in an unhealthy direction by increasing the abundance of proinflammatory genera (*Lactococcus* and *Leuconostoc*) while reducing those considered health promoting (*Allobaculum* and *Oscillospira*). These obesity-induced changes were antagonized by both Calanus oil (expressed as an apparent overrepresentation of *Lactobacillus* as well as underrepresentation of *Bilophila*) and exenatide (prevention of the HFD-induced increase in *Lactococcus* and decrease in the abundance of *Streptococcus*).

Chapter 5:

Discussion

5. Discussion

In this thesis, we confirmed previous findings (Hafstad et al. 2013) that high-fat feeding leads to a shift in myocardial metabolism, characterized by elevated fatty acid oxidation, while glucose oxidation is significantly reduced. We show for the first time (paper I) that dietary supplementation with Calanus oil during high-fat feeding is able to prevent this shift in myocardial energy utilization, similar to that achieved with infusion of the anti-diabetic drug, exenatide. Furthermore, Calanus oil supplementation (but not administration of exenatide) was cardioprotective in the sense that it led to a significant improvement in functional recovery following an ischemic event. The cardioprotective effect of the oil was further documented in experiments with rat cardiomyoblast exposed to palmitate stress (paper II). In these experiments, hydrolysed wax ester from Calanus oil effectively protected the cells from palmitate-induced cell death by inhibiting or preventing ER stress and impairment of autophagic flux. In paper III of this thesis, we showed that the unfavorable changes in the gut microbiota in response to high-fat feeding can be antagonized by dietary supplementation with Calanus oil.

Nearly 50 years ago, the Danish researchers Bang and Dyerberg reported a low incidence of ischemic heart disease in the Inuit people of Greenland, which was associated with their diet of fish, whales and seals, containing high amounts of long chain omega-3 polyunsaturated fatty acids (n-3 LC-PUFAs), such as EPA and DHA (Dyerberg et al. 1975; Bang et al. 1976). Later, clinical trials reported that supplementation with EPA and DHA reduced cardiovascular risk (Simopoulos 2002) and the risk of sudden cardiac death (GISSI-Prevenzione 1999). Over the last decades, the benefits of EPA and DHA on human health has been well acknowledged (Todoric et al. 2006; Harris and Zotor 2019), although in some studies supplementation with these fatty acids did not reduce the rate of cardiovascular events in subjects at high cardiovascular risk (Roncaglioni et al. 2013), diabetic patients (Bowman et al. 2018), as well as in patients without cardiovascular disease (Manson et al. 2019).

The main dietary source of n-3 LC-PUFAs in man is fish and seafood, but humans can also produce them from dietary a-linolenic acid (Bradbury 2011). Calanus oil has a relative low content of EPA and DHA, but unlike other marine oils, it contains large amounts of SDA, which is converted to EPA in the intestine. In addition, the oil contains mono-unsaturated fatty acids and fatty alcohols, which gives Calanus oil a unique chemical composition and probably explains its strong anti-inflammatory and anti-obesity effect (Pedersen, Vang, et al. 2014).

In contrast to previous studies from our research group (Salma et al. 2016; Höper et al. 2013; Höper et al. 2014), we did not see a clear effect of Calanus oil (2% dietary supplementation) on body weight development during high-fat feeding in this thesis (paper I and III), although it resulted in a significant reduction of the adiposity index (the sum of the individual intra-abdominal fat depots in grams). This apparent discrepancy can, most likely, be explained by the fact that we treated the mice with Calanus oil for a much shorter period (8 weeks), compared to the study by Höper et al. (Höper et al. 2014), in which the mice were treated for 27 weeks. The body weight development in our study was also in line with the study by Pedersen et al. (Pedersen, Salma, et al. 2014) who treated mice with 2% of Calanus oil over an 11 week period. It is also important to note that while previous studies have used male, the studies in paper I and paper III used female mice. Female mice are subjected to shifts in their hormonal status which can impact weight development (Grove et al. 2010) during different conditions. Furthermore, inflammation markers are completely differently expressed in female mice than in male mice (Grove et al. 2010).

The recordings of ventricular function following global ischemia showed some interesting results. First, we observed that the functional recovery of hearts from the high-fat diet (HFD) fed mice was not compromised, relative to that of the lean controls. On the contrary, HFD hearts recovered better than hearts from the lean controls. In light of several reports in the literature (Lund et al. 2015; Thakker et al. 2008; Wong et al. 2004), we were surprised by these observations, but it appears that

increased resistance to ischemic heart injury has been reported previously both in rats on a HFD (Wilson et al. 2007; Salie et al. 2014) and humans (Doehner et al. 2013). It should be emphasized that the mice used in this study were only mildly obese, and therefore the results could probably reflect the obesity paradox (see introduction). In line with this notion, Edland et al. (Edland et al. 2016) showed that long-term consumption of an obesogenic high-fat diet in female mice increased the tolerance to ischemia-reperfusion injury as expressed by reduced infarct size in *ex vivo* perfused hearts.

The second, and more important, finding in this study was that post-ischemic functional recovery of hearts from mice fed Calanus oil-supplemented HFD was superior, not only to hearts from the group receiving non-supplemented HFD, but also to hearts from the lean controls and mice treated with exenatide. We have no mechanistic explanation for this observation but, clearly, Calanus oil has a cardio-protective effect. One possibility could be that the improvement in glucometabolic control in response to Calanus oil supplementation resulted in a higher tolerance to ischemia (more efficient energy production), but low recovery of hearts from exenatide-treated hearts, which also showed improved glucometabolic control, makes this possibility less likely. We can therefore only speculate on alternative explanations, for instance that incorporation of n-3 LC-PUFAs into the sarcolemma might have improved membrane characteristics, causing inhibition of inflammatory signaling pathways, as well as modulation of cardiac ion channels and downstream cell signaling pathways, which could translate into improved post-ischemic recovery.

We were not able to follow up on the possible mechanisms behind the cardioprotective effect of Calanus oil by further studies in our mouse model, since the animal facility closed down in the spring of 2016 in connection with expansion/renovation of the unit. The expansion/renovation work was supposed to last for one year, but unexpected technical difficulties resulted in a more than four-year closedown period. Throughout this period, we were repeatedly told by the faculty and university administration that reopening was just around the corner. Because of this, and with support from the

Advanced Microscopy Platform at the Faculty, we switched to cell-based experiments, using the well-described cell line of rat neonatal cardiomyoblast (H9c2 cells) (paper II).

The most common saturated long-chain fatty acid, palmitate, is known to cause lipotoxicity and death in H9c2 cells (Yang et al. 2013; Park et al. 2015; Wei et al. 2013; Zou et al. 2017; Wu et al. 2019). This was confirmed in our investigations, both by live cell imaging and xCELLigence biosensor technology, which showed that cell death started to occur after approximately 8 h when incubated in the presence of 100 µmol/L palmitate. As H9c2 cells lack the enzymatic machinery to split the wax ester, the major lipid class in Calanus oil, we had to extract the wax ester from the oil and subsequently subject it to chemical hydrolysis to liberate the constituent fatty acids and fatty alcohols (see methods). The major finding was that co-incubation with hydrolysed wax ester from Calanus oil effectively prevented palmitate-induced cell death in a dose-dependent manner. Thus, even very low concentrations (below 10 µmol/L) of the hydrolysate significantly improved cell viability - a finding which supported the cardioprotective effect of Calanus oil observed in paper I. The fatty acid cocktail of Calanus oil contains poly-unsaturated fatty acids, such as EPA, DHA and particularly SDA, which are widely regarded as cardioprotective (Rimm et al. 2018; Hu et al. 2019). It is reasonable, therefore, to suggest that these fatty acids provide the cardioprotective effect mentioned above, but it cannot be excluded that the fatty alcohols can contribute as well (Sharma et al. 2019).

Palmitate is reported to induce cellular lipotoxicity with production of reactive oxygen species (ROS), impair cellular autophagy and induction of ER stress which leads to apoptotic cell death (Yang et al. 2019; Park et al. 2015; Jaishy et al. 2015). The results obtained in paper II suggest that ER stress and impaired autophagic flux is involved in palmitate-induced cell death, and that the hydrolysate of wax esters from Calanus oil prevented or inhibited these processes, suggesting a novel cardioprotective potential of this oil. Further studies are, however, needed to fully reveal the mechanisms involved.

In the third paper we asked ourselves whether the anti-obesity action of Calanus oil could prevent or antagonize unfavorable alterations of the intestinal microbiome in response to diet-induced obesity. The question is highly relevant because circumstantial evidence indicates that obesity leads to alterations in the gut microbiome (Ley et al. 2005), which in turn impact negatively on cardiometabolic function via activation of inflammatory processes (Honda and Littman 2016).

Our results confirmed previous reports (Ravussin et al. 2012; Hamilton et al. 2015; Costantini et al. 2017) that long-term feeding on a HFD changed the microbiota composition in an unhealthy direction, as reflected by an enrichment of the pro-inflammatory *Lactococcus*, as well as a depletion of the anti-inflammatory and health-promoting *Allobaculum* and *Oscillospira*. Although supplementing the HFD with Calanus oil resulted in a relatively high abundance of *Lactobacillus*, which is regarded a health-promoting genus and often related to weight loss (Costantini et al. 2017), it did not restore the microbiota composition seen in normal chow-fed lean mice. On the other hand, administration of the GLP-1 receptor agonist, exenatide, partly restored the bacterial profile found in the lean group, reducing the abundance of *Streptococcus* and *Lactococcus* which are considered pathogenic.

We did not detect any effect of Calanus oil supplementation or exenatide administration on inflammatory genes, neither in adipose tissue nor in the colon wall. In contrast, Caesar et al. (Caesar et al. 2015) reported reduced toll-like receptor 4 (TLR4)–induced secretion of monocyte chemoattractant protein-1 (MCP-1/CCL2) after treatment with fish oil. This discrepancy could be due to differences in the type of oil in question, or to differences in dosage and treatment period. Hence, the present results do not rule out the possibility that the influence of Calanus oil on the gut microbiota could have modified the activity of inflammatory cells in the intestine and in this way contributed to the improvement in cardiometabolic health as observed in diet-induced mice. Anyway, the number of various bacteria in the intestine is immense, and their relative abundance

vary a lot, so we find it difficult to make firm conclusions regarding possible health benefits based on the current selection of phyla/genera.

Concluding remarks

This thesis has shown that that dietary Calanus oil is able to prevent unfavorable alterations in myocardial metabolism which otherwise occur during obesity/nutrient stress. In addition, this oil protects the heart against ischemia-reperfusion damage. The mechanisms involved are not clear, but the current results suggest that fatty acids and/or fatty alcohols from Calanus oil-derived wax ester can protect cardiomyocytes from palmitate-induced lipotoxicity and reduce the consequent of ER stress and the impairment of autophagic flux. Finally, Calanus oil can attenuate some of the unfavorable alterations in the composition of the gut microbiota caused by obesity. Despite these results, more studies are needed to predict the mechanisms involved in this cardiac protection.

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Paper I:

Dietary Calanus oil recovers metabolic flexibility and rescues postischemic cardiac function in obese female mice

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RESEARCH ARTICLE | *Energetics and Metabolism*

Dietary Calanus oil recovers metabolic flexibility and rescues postischemic cardiac function in obese female mice

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Jansen KM, Moreno S, Garcia-Roves PM, Larsen TS. Dietary Calanus oil recovers metabolic flexibility and rescues postischemic cardiac function in obese female mice. *Am J Physiol Heart Circ Physiol* 317: H290–H299, 2019. First published May 24, 2019; doi:10.1152/ajpheart.00191.2019.—The aim of this study was to find out whether dietary supplementation with Calanus oil (a novel marine oil) or infusion of exenatide (an incretin mimetic) could counteract obesity-induced alterations in myocardial metabolism and improve postischemic recovery of left ventricular (LV) function. Female C57bl/6J mice received high-fat diet (HFD, 45% energy from fat) for 12 wk followed by 8-wk feeding with nonsupplemented HFD, HFD supplemented with 2% Calanus oil, or HFD plus exenatide infusion (10 $\mu g \cdot kg^{-1} \cdot day^{-1}$). A lean control group was included, receiving normal chow throughout the whole period. Fatty acid and glucose oxidation was measured in ex vivo perfused hearts during baseline conditions, while LV function was assessed with an intraventricular fluid-filled balloon before and after 20 min of global ischemia. HFD-fed mice receiving Calanus oil or exenatide showed less intra-abdominal fat deposition than mice receiving nonsupplemented HFD. Both treatments prevented the HFD-induced decline in myocardial glucose oxidation. Somewhat surprising, recovery of LV function was apparently better in hearts from mice fed nonsupplemented HFD relative to hearts from mice fed normal chow. More importantly however, postischemic recovery of hearts from mice receiving HFD with Calanus oil was superior to that of mice receiving nonsupplemented HFD and mice receiving HFD with exenatide, as expressed by better pressure development, contractility, and relaxation properties. In summary, dietary Calanus oil and administration of exenatide counteracted obesity-induced derangements of myocardial metabolism. Calanus oil also protected the heart from ischemia, which could have implications for the prevention of obesity-related cardiac disease.

NEW & NOTEWORTHY This article describes for the first time that dietary supplementation with a low amount (2%) of a novel marine oil (Calanus oil) in mice is able to prevent the overreliance of fatty acid oxidation for energy production during obesity. The same effect was observed with infusion of the incretin mimetic, exanatide. The improvement in myocardial metabolism in Calanus oil-treated mice was accompanied by a significantly better recovery of cardiac performance following ischemia-reperfusion.

Listen to this article's corresponding podcast at https://ajpheart.podbean.com/e/dietary-calanus-oil-energy-metabolism-and-cardiac-function/.

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ischemia-reperfusion; myocardial fatty acid oxidation; myocardial glucose oxidation; obesity; ventricular function

INTRODUCTION

Diabetes and diet-induced obesity are characterized by elevated supply and uptake of fatty acids to the heart, leading to a shift in myocardial energy metabolism toward fatty acid oxidation at the expense of glucose (1, 4, 23, 34). The rate of fatty acid uptake, however, may exceed the capacity for fatty acid oxidation, resulting in accumulation of lipid intermediates (triacylglycerol, diacylglycerol, ceramides) and reactive oxygen species which, over time, will create a lipotoxic state and impair myocardial metabolism as well as ventricular function (41).

Recent reports suggest that dysregulation of adipose tissue metabolism, in particular of the intra-abdominal fat depots, plays a central role in linking obesity to impairment of cardiac metabolism and function (7). A key finding is that adipocyte hypertrophy in response to nutrient stress creates a local low-grade inflammatory response with production of proinflammatory cytokines [tumor necrosis factor-α (TNFα), IL-6, and IL-1β] and chemokines (14, 19–21). Adipose tissue inflammation can also result in elevated serum levels of inflammatory cytokines (13), which in turn leads to activation of IKKβ/NF-κB and c-Jun-NH_2-terminal kinase (JNK) pathways and dysregulation of insulin action in peripheral tissues such as liver and skeletal muscle. The central role of abdominal fat in this process is probably due to its high lipolytic activity and direct drainage of inflammatory molecules and fatty acids to the liver via the portal vein (18). In fact, abdominal obesity has been regarded as the most serious new risk factor for cardiovascular and metabolic complications.

Of particular interest for this paper, Park et al. (32) reported that chronic high-fat feeding and obesity in mice impairs myocardial glucose metabolism, which was associated with ventricular hypertrophy and cardiac dysfunction. The same group reported that diet-induced obesity in mice increased macrophage and cytokine levels in heart, which was associated with significant reductions in AMPK phosphorylation and downregulation of glucose metabolism (25). The inflammatory response in obese adipose tissue is believed to be triggered by local hypoxia and activation of hypoxia-inducible factor (HIF)-1α (39), because, as the adipocytes expand and become hypertrophic, the distance between the blood-bearing vessels

increases and diffusion of oxygen becomes limited. Over time, this condition leads to local fibrosis and necrosis of adipocytes (39), which ultimately lead to inflammation and metabolic dysfunction, including increased mobilization of the fat stores (3, 24, 26). Therefore, the obvious solution to prevent adipose tissue inflammation and the accompanying metabolic and cardiovascular complications is to apply strategies for targeted reduction of this particular fat store in obese subjects. We (19, 20) have previously reported that dietary supplementation with a small amount of oil from the marine crustacean *Calanus finmarchicus* reduces both intra-abdominal and hepatic fat deposition while at the same time exerting a strong anti-inflammatory action in adipose tissue during high-fat feeding in male C57bl/6J mice. The main purpose of this paper was to find out whether these beneficial effects of Calanus oil also translate into improved myocardial metabolism and cardiac function in diet-induced obese (DIO) mice. For comparative reasons, we also tested the effect on these parameters of the glucagon-like peptide 1 (GLP-1) receptor agonist exenatide (marketed as Byretta), which is reported to increase insulin release from the pancreas and lower plasma glucose in diabetic patients (30).

MATERIALS AND METHODS

Animals and study design. The experiments were approved by the local authority of the National Animal Research Authority in Norway (FOTS id 8430), and the mice were treated according to the guidelines on accommodation and care of animals formulated by the European Convention for the Protection of Vertebrate Animals for Experimental and Other Scientific Purposes (Directive 2010/63/EU). The animals were housed at 23°C (3 animals per cage) under a reversed light-dark cycle (12-h:12-h dark-light) with ad libitum access to food and drinking water. Body weight and food intake were recorded once every week.

Seven week-old C57Bl/6J female mice (Charles River, Sulzfeld, Germany) were randomly divided into four groups (16 mice each), one receiving normal chow diet (NCD) containing 10% energy from fat (no. 58Y2, Test Diet; IPS, Notts, UK), whereas the other three groups received a lard-based high-fat diet (HFD) containing 45% energy from fat (no. 58V8, Test Diet, IPS, https://doi.org/10.6084/m9.figshare.8040137). After an initial 12-wk feeding period, the diet was replaced by HFD supplemented with 2% Calanus oil for one of the high-fat-fed groups (HFD + Cal, https://doi.org/10.6084/m9.figshare.8040143), while another (HFD + Ex) received 10 $\mu g \cdot kg^{-1} \cdot day^{-1}$ of the incretin mimetic exenatide (Polypeptide Laboratories, Ambernath, India), via miniosmotic pumps (Alzet Micro-Osmotic Pump model 1004; DURECT, ALZET Osmotic Pumps, Cupertino, CA). This feeding regimen continued for another 8 wk, so that the total feeding period lasted 20 wk.

The first 3–4 days after surgery and insertion of miniosmotic pumps, the mice were singly housed to secure healing of the operation wound. This was also the reason we used female mice, which are less aggressive than male mice. Also, mice that did not undergo surgery were subjected to single housing. Temgesic analgesic (0.1 mg/kg) was given 8 and 20 h postoperatively. A few mice were classified as low responders to the HFD (mice that did not increase their body weights above that of the lean controls) or high responders (mice whose body weights exceeded 40 g). These mice were excluded from the study. In addition, a few hearts were lost during perfusion due to technical problems.

Heart perfusion and recording, substrate oxidation, and ventricular function. The mice were anesthetized with pentobarbital sodium (100 mg/kg, 300 μl ip) mixed with heparin (100 U). Hearts were rapidly excised and placed in ice-cold Krebs-Henseleit bicarbonate buffer (KHB), containing (in mmol/l) 118.5 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.25 $CaCl_2$, 25.0 $NaHCO_3$, and 11.1 glucose. The aorta was immediately cannulated, and the hearts were retrogradely perfused with KHB, gassed with 95% O_2-5% CO_2 (pH 7.4, 37°C), under a pressure of 73.5 mmHg. A small fluid-filled balloon connected to a pressure transducer (Transpac IV; Abbott, North Chicago, IL) was inserted into the left ventricle via the mitral valve, and pressure signals were amplified and recorded by locally designed software (LabVIEW based). Thereafter, the hearts were connected to a buffer reservoir containing KHB supplied with 0.2 mmol/l palmitate bound to 3% BSA (fatty acid free; Europa Bioproducts, Cambridge UK) and perfused in recirculating mode.

After a 10-min stabilization period, the perfusion system was closed, and rates of glucose and fatty acid oxidation were determined simultaneously during the next 30 min by measuring $^{14}CO_2$ released from oxidation of [U-^{14}C]glucose and ^3H_2O released from oxidation of [9,10-^{3}H]palmitate, respectively, as described previously (2, 4, 5, 31). During this period, we also recorded preischemic values of left ventricular (LV) function.

The hearts were next subjected to 20 min of no-flow ischemia followed by 90 min of reperfusion. Left ventricular end-diastolic pressure (LVEDP) was measured during both ischemia and reperfusion and is given in millimeters of mercury. Recovery of other functional parameters was recorded and expressed as percentage of the corresponding preischemic values. At the end of reperfusion, hearts were frozen at −20°C and cut in slices of 1 mm thickness and stained with 1% 2,3,5-triphenyl-2H-tetrazolium chloride solution. Infarct size was calculated using ImageJ software (National Institutes of Health, Bethesda, MD).

Blood and tissue samples. Blood was collected (before excision of the heart) by puncture of the saphenous vein, and tissue samples and organs were taken immediately after excision of the heart for later analyses of blood lipids and mRNA expression.

Quantitative real-time PCR. RNA isolation was performed using quantitative reversed real-time PCR (qPCR). Perirenal white adipose tissue (WAT) samples were immersed in Allprotect Tissue Reagent (Qiagen) overnight at 4°C; 90–110 mg of tissue was used for RNA extraction in accordance with the RNeasy Lipid Tissue kit protocol (Qiagen). RNA concentrations were measured by use of Nanodrop and stored at −80°C before cDNA was prepared. cDNA was subsequently made according to a High Capacity cDNA reverse transcriptase kit (Thermo Fisher Scientific, Walthman, MA). cDNA was stored at −20°C until qPCR was performed in a Roche LightCycler 96, using a 1:5 dilution of the cDNA and the fast-start essential DNA green master (Roche, Basel Swiss). Five housekeeping genes were analyzed to normalize the expression of the target genes to the geometric mean of the two best housekeeping genes, which were selected on the basis of the average expression stability values determined with geNorm. For quantification of the gene expression in the perirenal WAT, we used hydroxymethylbilane synthase (HMBS) and Cyclo. Forward and reverse primers of the target genes analyzed in the perirenal WAT are shown in Supplemental Table S1 (available online at https://doi.org/10.6084/m9.figshare.7886621).

High-resolution respirometry. Mitochondrial function was assessed by high-resolution respirometry (Oroboros Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria). All respirometry experiments were performed on fresh heart tissue. Following excision of the heart, a piece of cardiac tissue was cut out of the left ventricle, washed, and stored in relaxing and biopsy preservation solution (BIOPS), containing (in mmol/l) 2.8 Ca_2K_2 EGTA, 7.2 K_2 EGTA, 5.8 ATP, 6.6 $MgCl_2$, 20 taurine, 15 Na_2 phosphocreatine, 20 imidazole, 0.5 dithiothreitol, and 50 MES 50 (pH 7.1). The tissue was thereafter homogenized using a PBI shredder SG3 (Pressure BioSciences, South Easton, MA) to a final concentration of 0.8 mg/ml in mitochondrial respiration medium (MiR05; containing in mmol/l: EGTA 0.5, $MgCl_2$ 3, K-lactobionate 60, KH_2PO_4 10, HEPES 20, and sucrose 110), pH 7.1. Mitochondria respiration was measured in the presence of several

substrates, as previously described by Cantó and Garcia-Roves (6) The O_2 flux that was left after adding antimycin A (residual oxygen consumption) was subtracted for the values of each step. Normalized flux ratios were calculated by dividing each value by the maximum flux.

Fatty acid composition of red blood cell membranes. Fatty acid composition of red blood cell (RBC) membranes was determined after methylation, as described by Hahn and Christie (17). The fatty acid methyl esters (FAMEs) were analyzed by capillary GLC using an Agilent 6890N (Agilent Technologies, Santa Clara, CA) gas chromatograph with a 50 m × 0.25 mm Chrompack CP-Sil 88 CB capillary column (Varian, Palo Alto, CA). The content of the individual fatty acids in the samples was expressed in percent total fatty acid content.

Statistical analysis. Data are presented as means ± SE. Graphs and statistical analyses were done in GraphPad Prism (GraphPad Software, San Diego, CA). Significant differences between treatment groups were assessed by one-way ANOVA followed by Dunnett's post hoc test. A P value <0.05 was considered statistically significant.

RESULTS

Administration of exenatide (but not dietary supplementation with Calanus oil) resulted in lower weight gain in mice on HFD compared with that of mice on HFD alone. We (36) previously reported that feeding young male mice HFD supplemented with 2% Calanus oil over an 8-wk period resulted in a slightly reduced body weight gain compared with that of mice given HFD alone. In the present study, we used adult female mice, which were made obese through an initial 12-wk feeding period on HFD. In this case, dietary Calanus oil had no effect on body weight gain. On the other hand, administration of exenatide resulted in a nearly 30% lower weight gain ($P < 0.05$) relative to the untreated HFD group (Fig. 1). In this group, we also noted a small temporary drop in body weight during the first week of treatment, which we assume was due to the surgery, since a similar drop in body weight was recorded in a few mice receiving saline-filled pumps (data not shown). There were no differences between the HFD groups with respect to organ weights (heart, liver, kidney, and spleen; Supplemental Fig. S1; https://doi.org/10.6084/m9.figshare.7886621), and food intake (Supplemental Fig. S2; https://doi.org/10.6084/m9.figshare.7886621) was similar for all HFD groups.

The reduced body weight gain in exenatide-treaded mice was reflected in reduced deposition of intra-abdominal fat, equivalent to a 35% ($P < 0.05$) reduction in the adiposity index. In the Calanus oil group, the adiposity index was reduced by 22% ($P < 0.05$), mainly due to reductions in perirenal and excess intra-abdominal fat (Fig. 2).

Dietary Calanus oil as well as exenatide administration prevented obesity-induced alterations in myocardial substrate oxidation. Following euthanasia at the end of the 8-wk treatment period, hearts were excised and perfused during baseline, normoxic conditions for measurement of myocardial substrate oxidation. In line with previous results (16), fatty acid oxidation was significantly increased ($P < 0.05$) in HFD mice at the expense of glucose oxidation (Fig. 3). Both dietary Calanus oil and exenatide administration, however, counteracted the obesity-induced switch in myocardial metabolism, leading to full recovery of the capacity for glucose oxidation without having any clear effect of fatty acid oxidation. (Fig. 3). There was no

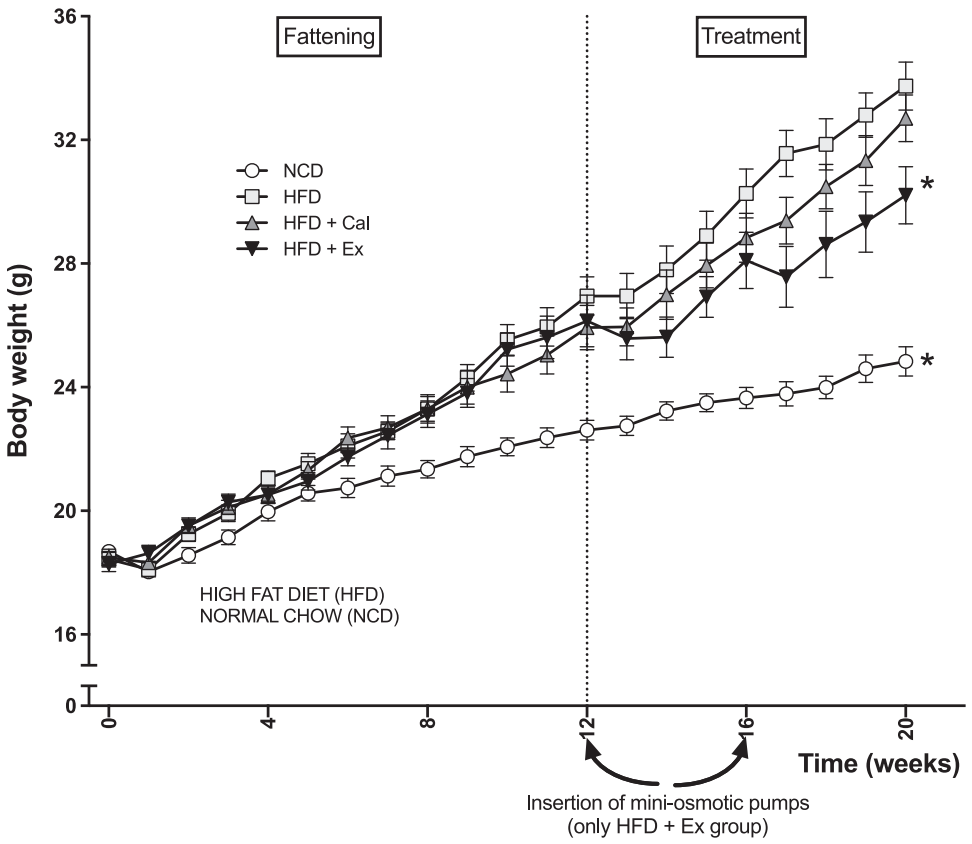

Fig. 1. Body weight development in high-fat diet (HFD)- and normal chow diet (NCD)-fed mice during the initial 12 wk feeding period as well as during the subsequent 8-wk treatment period. HFD and NCD groups stayed on their diet for the full 20 wk and served as lean and obese controls, respectively. After the 12-wk fattening period, 2 groups were treated with Calanus oil (HFD+Cal) or exenatide (HFD+Ex) for another 8 wk. Calanus oil was mixed into pellets by the manufacturer (TestDiet); exenatide was administered via miniosmotic pumps. *$P < 0.05$ vs. HFD (n = 10–15). Significant differences between treatment groups were assessed by two-way repeated-measures ANOVA.

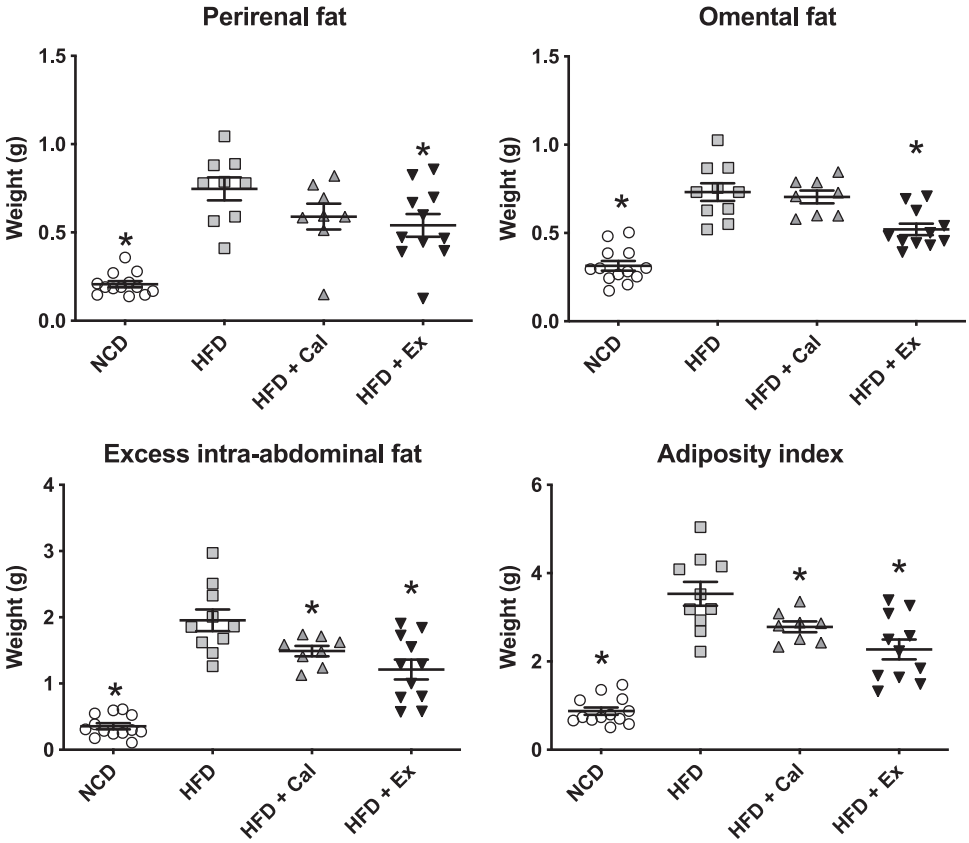

Fig. 2. Weight of different intra-abdominal fat depots at euthanasia of the various groups of mice. NCD, normal chow diet; HFD, high-fat diet; HFD+Cal, HFD + Calanus oil; HFD+Ex, HFD + exenatide. Adiposity index was calculated as the sum of the individual fat depots. *$P < 0.05$ vs. HFD (n = 8–13). Significant differences between treatment groups were assessed by one-way ANOVA, followed by Dunnett's post hoc test.

difference between the two treatments regarding their impact on myocardial substrate oxidation.

Dietary Calanus oil, but not exenatide administration, rescued myocardial ischemia-reperfusion injury. To test whether the improvements in myocardial energy metabolism had any cardioprotective correlate, hearts from the various groups were subjected to ischemia-reperfusion (20-min no-flow ischemia followed by 120-min reperfusion). Preischemic functional parameters are given in Table 1, indicating slightly better pressure development, as well as inotropic (dP/dt_{max}) and lusitropic (dP/dt_{min}) states in hearts from all HFD groups relative to the lean controls. None of these differences, however, were statistically significant.

The ischemic insult produced a marked increase in the intraventricular pressure, plateauing at values around 50 mmHg, again with no differences between the groups (Fig. 4). A postischemic peak in the LVEDP was recorded 5 min after start of reperfusion, but again there were no differences in the peak values or rate of decline of LVEDP throughout the reperfusion period.

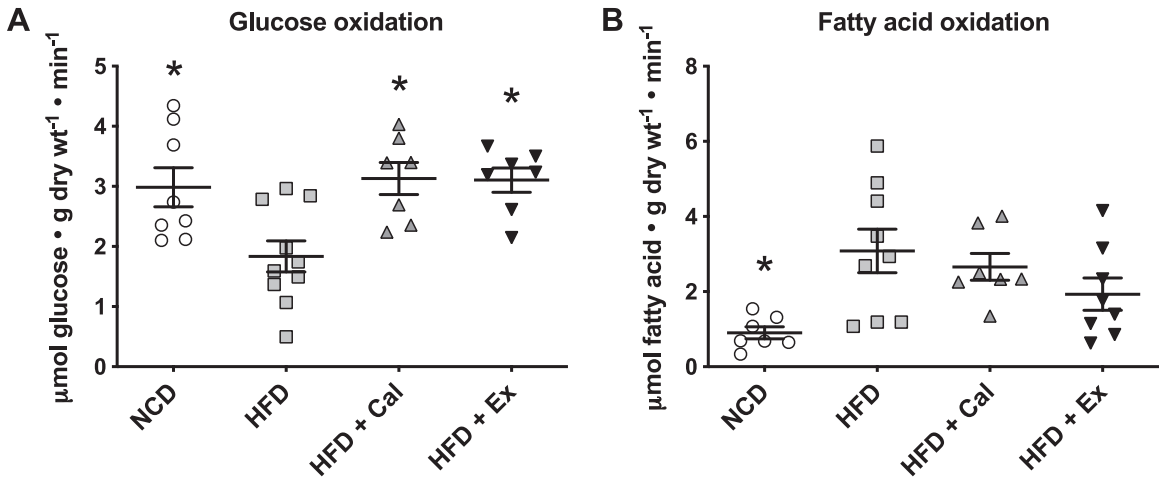

Fig. 3. Myocardial glucose (*A*) and fatty acid (*B*) oxidation in the various groups of mice during baseline normoxic perfusion. NCD, normal chow diet; HFD, high-fat diet; HFD+Cal, HFD + Calanus oil; HFD+Ex, HFD + exenatide. *$P < 0.05$ vs. HFD (n = 7–10). Significant differences between treatment groups were assessed by one-way ANOVA followed by Dunnett's post hoc test.

Table 1. *Preischemic left ventricular function during baseline, normoxic conditions*

	NCD	HFD	HFD+Cal	HFD+Ex
n	9	11	7	9
LVSP	121 ± 12	145 ± 6	135 ± 8	150 ± 6
LVDevP	109 ± 12	134 ± 6	124 ± 8	139 ± 7
LVEDP	15 ± 1	14 ± 1	15 ± 1	15 ± 1
dP/dt_{max}	4,493 ± 500	5,818 ± 263	5,340 ± 376	5,762 ± 281
dP/dt_{min}	−3,133 ± 391	−4,219 ± 263	−3,698 ± 364	−3,988 ± 224
Heart rate, beats/min	279 ± 11	289 ± 12	279 ± 20	291 ± 15

Values are means ± SE and are based on the 3 last recordings before ischemia. Ventricular function was assessed using a fluid-filled balloon in the left ventricle. NCD, normal chow diet; HFD, high-fat diet; HFD+Cal, HFD + Calanus oil; HFD+Ex, HFD + exenatide. LVSP, left ventricular systolic pressure; LVDevP, left ventricular developed pressure; dP/dt_{max}, maximum rate of pressure change during isovolumic contraction; dP/dt_{min}, minimum rate of pressure change during isovolumic relaxation.

In contrast to the prevailing view, we did not observe any negative impact of high-fat feeding on postischemic recovery of the other parameters of ventricular function [LV developed pressure (LVDevP), dP/dt_{max}, and dP/dt_{min}; Fig. 5]. The important finding, however, was that postischemic recovery of these functional parameters in the HFD group receiving Calanus oil was superior to that of the nontreated HFD group, as well as the HFD + Ex and lean control groups ($P < 0.05$). We also measured infarct size; however, the values were similar for all groups (including the lean control group), ranging between 47 and 58% (Supplemental Fig. S3; https://doi.org/10.6084/m9.figshare.7886621).

Effect of Calanus oil and exenatide on cardiac mitochondrial function. Differences in cardiac mitochondrial respiration between the various groups were studied in freshly dissected tissue from the left ventricle, using high-resolution respirometry. No statistically significant differences were observed between the groups for any of the respiratory states except for a slightly higher (nonsignificant) oxygen flux in the Calanus-oil group in the presence of complex I substrates relative to the other groups (Fig. 6*A*). This difference was also evident when the flux control ratios wer calculated for the different respiratory states (i.e., the relative contribution of each respiratory state to the maximum flux; Fig. 6*B*). Maximum respiration in the coupled state (following addition of glutamate and succinate), with electron input through both complexes I and II (CI+II) was not different between groups. Furthermore, oxygen flux was essentially unaltered following addition of the exogenous uncoupler FCCP, reflecting the efficiency of the phosphorylation system (adenine nucleotide translocase, phosphate transporter, and ATP synthase) in matching the potential of the electron transfer system in mouse cardiac muscle.

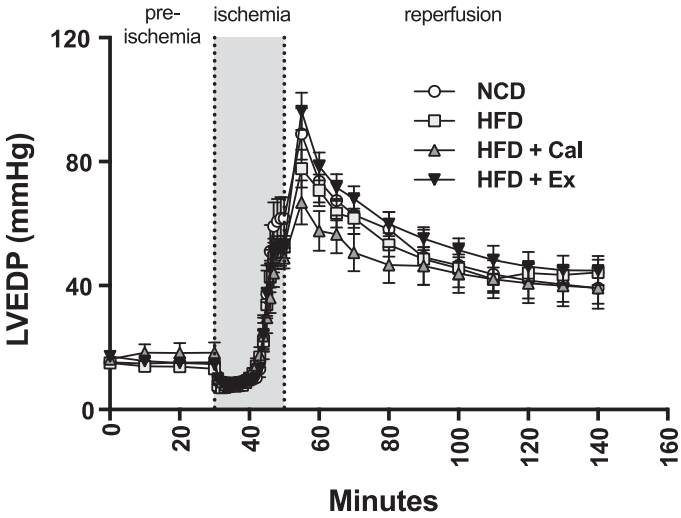

Fig. 4. Development of cardiac contracture during no-flow ischemia, as well as postischemic rise and decline in left ventricular end-diastolic pressure (LVEDP) in hearts from the various groups of mice. NCD, normal chow diet; HFD, high-fat diet; HFD+Cal, HFD + Calanus oil; HFD+Ex, HFD + exenatide. LVEDP during the normoxic, preischemic perfusion was set to 15 mmHg. No statistically significant differences were observed between treatment groups as tested by two-way repeated-measures ANOVA.

Fatty acid composition of RBC membranes. Gas chromatography analysis revealed significantly higher content of polyunsaturated ω-3 fatty acids in RBC membranes of mice receiving Calanus oil-supplemented HFD compared with that of the other HFD groups, as well as the lean control (NCD) group. This resulted in a marked increase in the ω-3 index and in the n-3/n-6 ratio (Table 2).

Gene expression. To find out whether the observed alterations in metabolism were reflected at the gene level, we examined mRNA expression of genes involved in metabolic regulation in adipose tissue. Accumulation of intra-abdominal fat in the HFD groups was associated with increased mRNA expression of CD36 in perirenal WAT relative to that of NCD-fed mice, in line with a high fatty acid uptake in the adipocytes (Fig. 4 and Supplemental data; https://doi.org/10.6084/m9.figshare.7886621). This response was not influenced, however, by Calanus oil supplementation or administration of exenatide. Expression of GLUT4 was somewhat lower in mice receiving Calanus oil, whereas expression of pyruvate dehydrogenase (PDH) kinase-4 (PDK4) was reduced in both the Calanus oil and exenatide groups, which might be a compensatory mechanism to maintain the flux through the PDH complex despite reduced glucose uptake.

Low-grade inflammation and release of proinflammatory adipokines in obese adipose tissue are suggested to cause insulin resistance in peripheral tissues. We found, however, that mRNA expression of proinflammatory genes like IL-6 and TNFα was extremely lowly expressed in perirenal adipose tissue (data not included), but other indicators of inflammation [monocyte chemoattractant protein-1 (MCP1) and EGF-like module-containing mucin-like hormone receptor-like 1 (EMR1), also known as F4/80) was significantly increased in the HFD group relative to NCD-fed mice. All three HFD groups showed increased expression of G protein-coupled receptor 120 (GPR120), and a very unexpected finding was that adi-

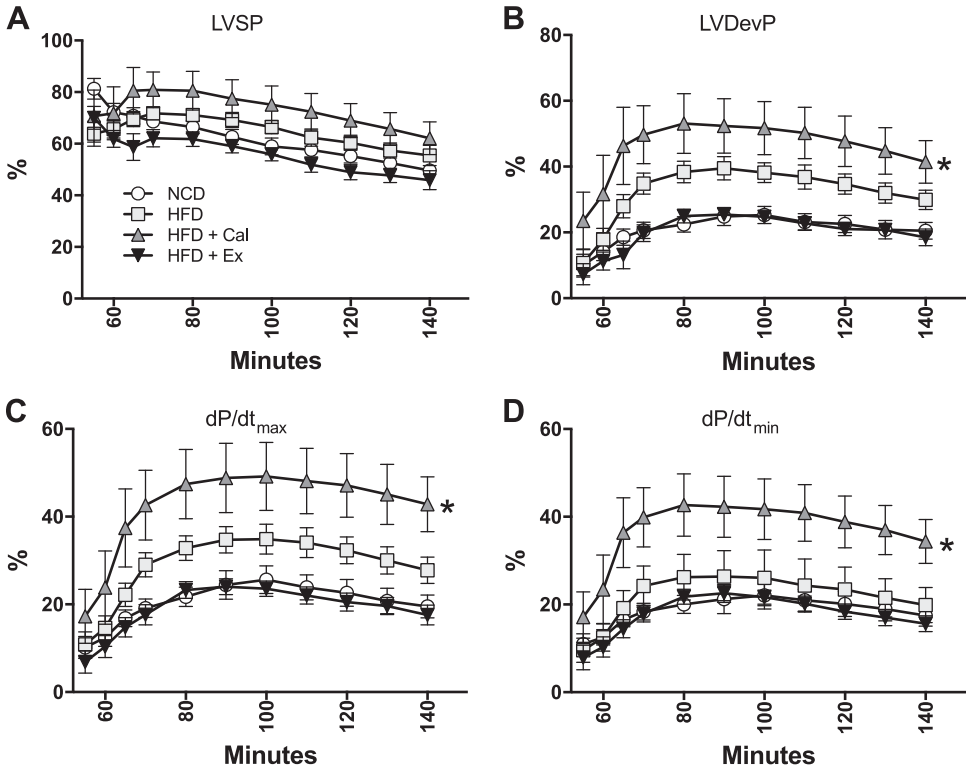

Fig. 5. Postischemic recovery of left ventricular (LV) function in ex vivo perfused hearts from the various groups of mice. NCD, normal chow diet; HFD, high-fat diet; HFD+Cal, HFD + Calanus oil; HFD+Ex, HFD + exenatide. *A*: LVSP, LV systolic pressure. *B*: LVDevP, LV developed pressure. *C*: dP/dt_{max}, maximum rate of pressure change during isovolumic contraction. *D*: dP/dt_{min}, minimum rate of pressure change during isovolumic relaxation. Heart rate recovered to ~80% of preischemic values in all groups (not shown). *$P < 0.05$ vs. HFD (n = 7–11 in each group). Area under the curve was calculated for each heart in the various groups. Significant differences between treatment groups were assessed by one-way ANOVA followed by Dunnett's post hoc test.

ponectin was significantly increased in the HDF groups relative to the lean control group.

DISCUSSION

Main findings. Obesity induced by obesogenic diets is characterized by a shift in myocardial energy metabolism toward increased fatty acid oxidation at the expense of carbohydrates. In the present study, however, we show that dietary supplementation with Calanus oil, as well as with administration of the GLP-1 receptor agonist exenatide (incretin mimetic), were able to prevent the obesity-induced decline in myocardial glucose utilization, while fatty acid utilization was not significantly affected. In contrast to the notion that obesity impairs recovery of cardiac function after an ischemic insult, we observed that the postischemic recovery of ventricular function in ex vivo perfused hearts from high-fat-fed mice was not

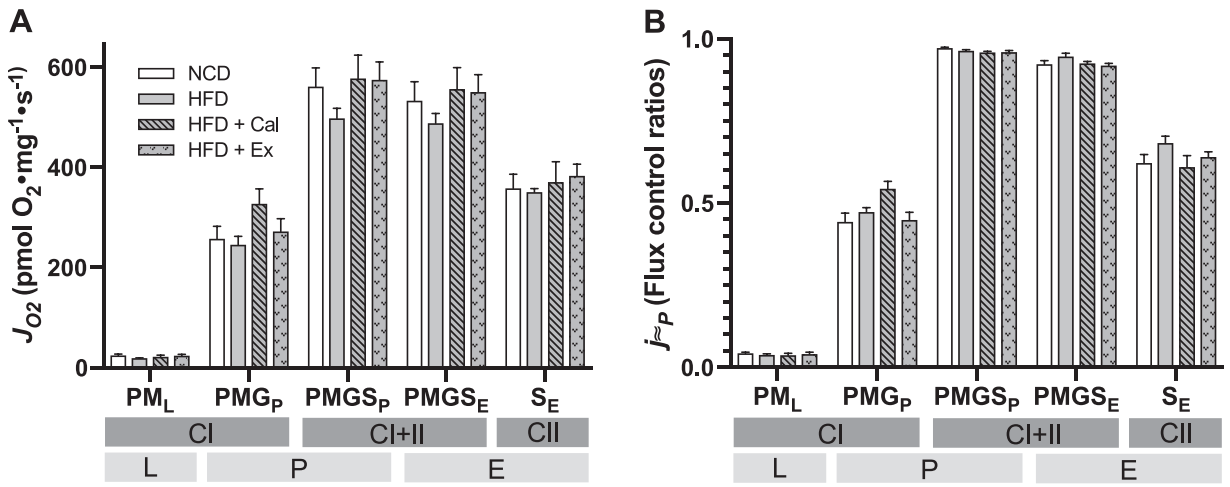

Fig. 6. Mitochondrial respiration in cardiac muscle at the end of the experimental period for the same group of mice as described in Fig. 1. Oxygen flux (J_{O2}) was measured with an Oroboros-2k oxygraph. *A*: first, pyruvate and malate (PM) were added for assessment of oxygen flux in the LEAK state (L). Thereafter, ADP, cytochrome *c*, and glutamate (G) were added to measure oxygen flux in the oxidative phosphorylation state (P) with electron flow from complex I (CI). Maximum coupled respiration with electron flow from both complexes I and II (CI+II) was obtained following addition of succinate (S). Electron transfer system (E) capacity was measured after addition of FCCP, followed by rotenone (complex I inhibitor) to determine the specific contribution from complex II (CII). Finally, antimycin A was added to inhibit complex III, and the remaining oxygen flux (residual oxygen consumption) was subtracted from each of the previous respiratory states. *B*: flux control ratios (j≈P), i.e., oxygen flux rates in the various respiratory states normalized to maximum flux rate; n = 8, 7, 7, and 10 for normal chow diet (NCD), high-fat diet (HFD), HFD + Calanus oil (HFD+Cal) and HFD + exenatide (HFD+Ex), respectively.

Table 2. *Fatty acid composition of red blood cell membranes (RBC)*

Fatty Acid	NCD	HFD	HFD+Cal	HFD+Ex
16:0	25.58 ± 0.33	23.87 ± 0.38	25.36 ± 0.36	23.98 ± 0.29
16:1n-7	1.24 ± 0.44	0.83 ± 0.23	0.87 ± 0.13	0.81 ± 0.11
18:0	13.66 ± 0.60	16.71 ± 0.93	16.26 ± 0.54	17.03 ± 0.76
18:1n-9	14.86 ± 0.46	12.95 ± 0.31	12.99 ± 0.25	13.08 ± 0.39
18:1n-7	2.41 ± 0.21	1.40 ± 0.11	1.31 ± 0.06	1.42 ± 0.11
18:2n-6	6.75 ± 0.47	9.41 ± 0.55	9.50 ± 0.20	8.17 ± 3.06
18:4n-3	ND	ND	0.55 ± 0.02	ND
20:4n-6	22.84 ± 0.62	22.92 ± 0.71	17.02 ± 0.28*	22.53 ± 0.50
20:5n-3	0.84 ± 0.12	0.71 ± 0.15	2.77 ± 0.38*	0.64 ± 0.08
22:4n-6	1.98 ± 0.09	2.45 ± 0.09	1.12 ± 0.07*	2.47 ± 0.09
24:1n-9	1.31 ± 0.07	1.21 ± 0.14	ND	1.17 ± 0.09
22:5n-3	0.55 ± 0.04	0.64 ± 0.06	1.66 ± 0.07*	0.61 ± 0.04
22:6n-3	7.96 ± 0.48	6.91 ± 0.49	10.72 ± 0.40*	7.09 ± 0.34
ω-3 index	10.1 ± 0.4	9.0 ± 0.7	18.5 ± 1.0*	9.2 ± 0.6
n-3/n-6 ratio	29.5 ± 1.6	23.7 ± 1.5	56.4 ± 2.6*	25.4 ± 3.2

Values are means ± SD. Relative percentages of various fatty acids in red blood cell (RBC) membranes from the various groups of mice. NCD, normal chow diet; HFD, high-fat diet; HFD+Cal, HFD + Calanus oil; HFD+Ex, HFD + exenatide; ND, not detected. Note significantly higher values of n-3 polyunsaturated fatty acids (PUFAs) and lower content of n-6 PUFAs in RBCs from HFD+Cal mice, resulting in a significantly higher ω-3 index and n-3/n-6 ratio for this group. Also, stearidonic acid (18:4n-3) was detected only in RBCs from the HFD+Cal group. *$P < 0.05$ vs. all other groups (n = 7–9).

impaired relative to hearts from mice receiving normal chow. More importantly, postischemic recovery of hearts from mice receiving HFD with Calanus oil exhibited significantly better recovery than hearts from mice on nonsupplemented HFD, indicating cardioprotective properties of the Calanus oil.

Antiobesogenic effect of Calanus oil and exenatide. High-fat feeding resulted in increased deposition of intra-abdominal fat (supported by increased mRNA expression of CD36 and GPR120). However, the results confirmed previous reports demonstrating that both dietary Calanus oil and administration of exenatide (30) have antiobesogenic effects, albeit less pronounced in the female mice used in the present study than in that previously reported for male mice (19, 20). The mechanism behind the antiobesogenic effect is so far unknown, and both current and previous results (19, 20) exclude the possibility that it is due to reduced energy intake. Mack et al. (30) reported, however, decreased food intake and a drop in body weight gain in DIO mice during the first week following administration of exenatide and claimed that this could have been due to the discomfort of the animals, since both emesis and nausea have been reported with clinical use of the drug. Food intake also dropped temporarily following infusion of exenatide in the current experiment, after both the first and second insertions of miniosmotic pumps. However, we believe that this response was due to the discomfort associated with the surgical procedure, since insertion of saline-filled miniosmotic pumps (in a few mice) showed a similar drop in body weight (not shown).

Effect of Calanus oil and exenatide on adipose tissue metabolism. Obesity is associated with increased adipose tissue lipolysis and increased release of fatty acids to the circulation due to increased size of the adipocytes as well as insulin resistance (3, 11, 26). Moreover, obesity is tightly associated with the development of a local low-grade inflammation in adipose tissue. Thus, expansion of adipocytes results in elevated production of inflammatory cytokines such as TNFα and IL-6 in obese individuals (21), which may negatively influence insulin action in adipocytes and hepatocytes via activation of IKKβ/NF-κB and JNK pathways (37). In contrast to previous results in male DIO mice (19, 20), mRNA expression of TNFα and IL-6 was hardly detectable in the current study on HFD-fed female mice (data not shown); the only evidence of obesity-induced inflammation was an apparent increase in the expression of MCP1 and EMR1, which was not influenced by Calanus oil or exenatide treatment. The low inflammatory status could probably be explained by the finding that HFD induced only a relatively mild degree of adiposity, so that the signal for adipokine secretion (39) was missing. In addition, it has been reported that genes involved in inflammation are more highly upregulated in males than in females (15). Also, the present observation of increased mRNA expression of adiponectin in adipose tissue in response to HFD is in line with previous reports (10). Still, dietary Calanus oil or infusion of exenatide resulted in reduced deposition of intra-abdominal fat compared with that of untreated HFD mice. The underlying mechanism is not clear, but increased adipose tissue lipolysis and/or decreased lipogenesis could be involved. In addition, increased hepatic uptake of fatty acids could drain fatty acids from the abdominal fat stores. However, these possibilities need to be further investigated.

Effect of Calanus oil and exenatide on myocardial metabolism. The high energy demand of the heart is covered to a large extent by oxidation of fatty acids. Obesity, however, leads to an imbalance between fatty acid uptake and oxidation, wherein the myocardial fatty acid supply exceeds the fatty acid oxidation capacity of the heart. The obesity-induced changes in myocardial substrate oxidation were confirmed in the female hearts used in the current experiments. Of note, however, dietary Calanus oil supplementation as well as exenatide administration were able to restore the ability of the heart to oxidize glucose but did not significantly suppress the myocardial overreliance on fatty acid oxidation. Thus, one gets the impression that the two treatments led to an increase in total myocardial substrate oxidation, but calculating the sum of ATP-production from fatty acids and glucose (38 ATP/mole glucose and 131 ATP/mole fatty acid) showed that the total ATP production was similar in the three high-fat-fed groups. Having said that, we have no information on any contribution from other substrates, e.g., ketone bodies).

Intuitively, one would expect that the reduction in fat mass (and probably also in hepatic fat content) in response to these treatments be reflected in reduced fatty acid delivery from adipose tissue (and of triacylglycerol from the liver) to the blood. Thus, the observed improvement in myocardial metabolism following these treatments could be explained in terms of the Randle effect (23, 34), where lower levels of circulating lipid substrates (fatty acid and triacylglycerol) render the heart less fatty acid dependent for energy production, while allowing recovery of the myocardial capacity for glucose oxidation. Measurements of plasma fatty acid and triacylglycerol concentrations, however, revealed no difference between the groups for any of these lipids (Supplemental Fig. S5; https://doi.org/10.6084/m9.figshare.7886621). Still, one should bear in mind that the observed concentrations of these lipid substrates represent merely spot measurements, which do not necessarily reflect the long-term supply of lipids to the heart. Also, there is reason to believe that suppression of adipose tissue inflamma-

tion and reduced release of adipose tissue-derived proinflammatory cytokines (19) play a role, because these substances can negatively influence metabolic pathways and insulin signaling in the heart (27).

Effect of Calanus oil and exenatide on postischemic recovery of ventricular function. Normally, one would expect that high-fat feeding leads to accumulation of myocardial triglyceride (TG), and that mobilization of these TG stores (catalyzed by adipose triglyceride lipase, ATGL) should release fatty acids, which, together with the external fatty acid supply, could lead to lipid overload and accumulation of toxic lipid metabolites. Thus, previous studies on rodent models (1, 28) have reported that obesity-induced shifts in myocardial fuel selection in favor of fatty acids are associated with accumulation of toxic lipid intermediates and contractile abnormalities (16, 22, 42). Moreover, Peterson (33) reported reductions in both systolic myocardial velocity and early diastolic myocardial velocity with increasing BMI in young, healthy, obese women. In the present study, using ex vivo perfused female mouse hearts, long-term feeding with HFD did not impair ventricular function. If anything, the functional parameters obtained during baseline normoxic conditions were indicative of improved performance of hearts from the high-fat-fed groups, although the effects were not statistically significant (Table 1). Furthermore, high-fat feeding did not compromise postischemic functional recovery, since average pressure development and contractility were not impaired relative to hearts from lean controls. If anything, HFD hearts recovered better than hearts from the lean controls. At first glance, and in light of several reports in the literature (29, 40, 44, 45), we were surprised by these observations, but a deeper analysis of the literature revealed that increased resistance to ischemic heart injury has been reported previously both in rats fed a HFD (35, 43), and in high-fat-consuming humans (the "obesity paradox") (8). Furthermore, a recent study by Edland et al. (9) showed that long-term consumption of an obesogenic diet in mice increased the tolerance to ischemia-reperfusion injury by reducing infarct size in ex vivo perfused hearts from these mice. Of note, the study by Edland et al., as well as the present study, used female mice, and it would be of interest to find out whether there are sex differences that might influence the outcome of an ischemic insult in obesity.

The explanation why postischemic recovery of hearts from mice fed Calanus oil-supplemented HFD was superior to that of the other groups is not clear. In particular, we need an explanation why hearts from mice treated with exenatide did not recover LV function to the same degree as hearts from Calanus oil-treated mice. The metabolic pattern before ischemia revealed that both treatments abrogated the obesity-induced suppression of glucose oxidation. Such an improvement in cardiac metabolism is expected to result in increased cardiac efficiency (22) and less accumulation of lipotoxic metabolites (12), which in turn would prime the hearts to better tolerate the ischemic insult and the subsequent stress during reperfusion. Therefore, the finding that hearts from Calanus oil-treated mice showed significantly better recovery of LV function than those from exenatide-treated mice (as well as the other groups), appears to be unrelated to the improvement in glucometabolic control. Probably, eight weeks of Calanus oil treatment might have led to neurohumoral and/or hemodynamic alterations that have the potential to change the intrinsic properties of the heart, which persist in ex vivo perfusions. Moreover, obesity influences more than just glucose and fatty acid oxidation, leaving the possibility that Calanus oil derived ω-3 fatty acids might have influenced additional aspects of myocardial metabolism, e.g., reduction of oxidative stress (38).

In an attempt to find out whether dietary Calanus oil supplementation was associated with improved myocardial energy production, we measured mitochondrial respiration in cardiac fibers, using a standard protocol. However, we were not able to detect any differences between the groups for any of the respiratory states except for a slightly (nonsignificant) elevated oxygen flux in the presence of complex I substrates. Alternatively, one might speculate whether Calanus oil (or its metabolites) has a direct effect on the contractile apparatus due to incorporation of ω-3 fatty acids into the sarcolemma, thereby modifying the membrane fluidity and improving calcium transport in the cardiomyocytes. In support of this hypothesis, we measured a significantly higher ω-3 index (as well as n-3/n-6 ratio) in RBC membranes from the Calanus oil-treated group (Table 2). Further studies are needed, however, to explain the beneficial impact of Calanus oil on postischemic contractile function in hearts from obese mice.

Conclusion. Obesity induced by high-fat feeding shifts myocardial substrate metabolism toward almost exclusively fatty acid oxidation at the expense of glucose. Both dietary Calanus oil and exenatide treatment counteracted these metabolic derangements. Calanus oil supplementation of the HFD provided, in addition, protection from ischemia-reperfusion damage, apparently unrelated to the concomitant improvement in myocardial metabolism.

Limitations. One limitation of this study was the lack of any assessment of insulin sensitivity or insulin signaling in response both to high-fat feeding and following treatment with Calanus oil and exenatide. The study would also have benefitted from assessments of inflammatory markers in plasma and/or cardiac tissue to suggest causality between the reported parameters. Finally, inclusion of fatty acids as respiratory substrate would have added additional information regarding the mitochondrial function in response to the treatments.

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DISCLOSURES

T. S. Larsen has a small position as scientific advisor in Calanus AS. None of the other authors has any conflicts of interest, financial or otherwise, to disclose.

AUTHOR CONTRIBUTIONS

K.M.J., P.M.G.-R., and T.S.L. conceived and designed research; K.M.J., S.M., P.M.G.-R., and T.S.L. performed experiments; K.M.J., S.M., P.M.G.-R., and T.S.L. analyzed data; K.M.J., P.M.G.-R., and T.S.L. interpreted results of experiments; K.M.J. and T.S.L. prepared figures; K.M.J., P.M.G.-R., and T.S.L. drafted manuscript; K.M.J., S.M., P.M.G.-R., and T.S.L. approved final version of manuscript.

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Paper II:

Hydrolysed wax ester from Calanus oil protects H9c2 cardiomyoblast from palmitate – induced lipotoxicity

Hydrolysed wax ester from Calanus oil protects H9c2 cardiomyoblasts from palmitate-induced lipotoxicity

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Short title: Hydrolyzed wax ester prevents lipotoxic stress

Key words: palmitic acid, lipotoxicity, ER stress, wax ester, PUFA

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Abstract

Lipotoxicity, induced by an excessive load of saturated fatty acids, plays an important role in the development of obesity-induced heart failure. Accordingly, palmitate-induced lipotoxicity has been linked to increased oxidative and endoplasmic reticulum (ER) stress, impaired autophagic flux and apoptotic cell death. Previous reports have shown that Calanus oil and purified wax ester from this oil have beneficial systemic and cardiac effects in diet-induced obese mice. Here we evaluated the effect of hydrolysed wax ester from Calanus oil (WE_H) on palmitate-induced ER stress and viability in H9c2 cells. Incubation of the cells with palmitate (100 µM) for 20 h resulted in approximately 80% cell death. Co-incubation with WE_H resulted in a dose-dependent increase in cell survival, where 10 µM WE_H almost completely abrogated palmitate-induced cell death. Furthermore, palmitate-induced ER stress, as reflected by a significant increase CHOP expression and nuclear translocation, was markedly reduced by co-incubation with WE_H. Palmitate-treated cells also displayed numerous enlarged LC3B-positive vesicles and p62-positive aggregates/inclusions, suggesting an impaired autophagic flux, which was markedly diminished by co-incubation with WE_H. In conclusion, these data demonstrate that WE_H protects H9c2 cells against palmitate-induced lipotoxicity via inhibiting or preventing ER stress and impairment of autophagic flux.

Introduction

Obesity and diabetes can result in excessive accumulation of lipids in the myocardium[1, 2] which, together with their intermediate products, causes a condition of lipotoxicity with disturbances of the cellular metabolism and development of cardiac dysfunction[3]. An excessive load of saturated fatty acids, plays an important role in the development of obesity-induced heart failure. Accordingly, palmitate-induced lipotoxicity has been linked to increased oxidative and endoplasmic reticulum (ER) stress, impaired autophagic flux and apoptotic cell death.

ER is an organelle that monitor and maintain cellular homeostasis, and lipotoxicity is reported to cause ER stress[4], e.g. after high-fat diet feeding in mice[5] and after saturated fatty acid treatment (such as palmitate) of H9c2 cells[6, 7]. Sustained ER stress plays a critical role in the development of cardiac dysfunction by contributing to apoptotic cell death[6, 8], and to handle this stress autophagy is activated to remove damaged organelles and mis/unfolded proteins[9, 10]. Although, nutrient-induced changes in autophagy seems to vary between cell types[11-16], palmitate has been reported to facilitates autophagosome accumulation in H9c2 cells[6, 17]. Moreover, several findings suggest that increased autophagy has a protective effect against cell death during cellular stress, while sustained activation may have detrimental effects[6, 18].

Interestingly, omega-3 poly-unsaturated fatty acids are shown to attenuate ER stress in primary rat hepatocytes[19] and has been suggested as a potential strategy to counteract obesity-induced lipotoxicity[20]. We have previously reported that dietary supplementation with a small amount of Calanus oil from the marine crustacean Calanus *finmarchicus* reduces intra-abdominal fat deposition, as well as adipose tissue inflammation in high-fat diet (HFD)-fed mice[21, 22]. More recently, we found that Calanus oil supplementation improved cardiac metabolism in HFD mice by alleviating the obesity-induced over-reliance on fatty acid oxidation, while at the same time restoring the capacity for glucose oxidation[23]. Of particular interest was the finding that hearts from mice receiving HFD supplemented with Calanus oil showed significantly better recovery of ventricular function following

an ischemic insult. The mechanism behind this cardioprotective effect of Calanus oil is, however, not clear. The purpose of this study was therefore to find out whether the unique fatty acid cocktail of Calanus oil, containing a variety of poly-unsaturated omega-3 fatty acids and mono-unsaturated fatty acids, could have any direct beneficial effects on the heart by examining its effect on palmitate-induced lipotoxic stress in H9c2 cells.

Material and methods

Cell culture

H9c2 cells (rat embryonic cardiomyoblasts, ECACC Cell Lines, Sigma-Aldrich, England) were cultured at 37°C with 5% CO_2 using Dulbecco's modified eagles medium high glucose (DMEM D5796, Sigma-Aldrich, St. Louis MO, USA), 10% fetal bovine serum (FBS, Sigma-Aldrich), 100 U/mL penicillin and 100 µg/mL streptomycin (p0781, Sigma-Aldrich). Cells from passage 8 to 15 were used in this study. To induce a lipotoxic stress, the cells were incubated with 100 µM palmitate (palmitic acid, P0500 Sigma-Aldrich) dissolved in ethanol for a total period of 20 h. Cells incubated in the presence of vehicle (ethanol) served as control. In addition, palmitate-exposed cells were co-incubated with hydrolyzed wax ester (0.2-10 µM) from Calanus oil. To inhibit and induce ER stress the cells were incubated with, salubrinal (60 µM, Sigma-Aldrich) and tunicamycin (2.5 µg/mL, Sigma-Aldrich), respectively. To inhibit and induce autophagy the cells were incubated with, LY294002 (20 µM, Sigma-Aldrich) and rapamycin (1 µM, Sigma-Aldrich) respectively.

Isolation and hydrolysis of wax ester from Calanus oil

Neutral lipids from Calanus oil were isolated by solid phase extraction, using five Mega Bond Elut aminopropyl SPE disposable columns (Varian Inc., Middelburg, Netherlands) mounted on a Visiprep vacuum manifold (Supelco, Bellefonte, PA, USA), as described by Vang et al. [24]. Following conditioning with heptane, each column was loaded with 80-100 mg Calanus oil (in 2 mL chloroform). The neutral lipids were eluted with chloroform: isopropanol (2:1), dried under a stream of nitrogen and re-dissolved in 1 mL heptane. The lipid extracts were loaded on new columns, and the wax esters subsequently eluted with heptane, dried and re-dissolved in chloroform. The wax esters were subsequently hydrolyzed in ethanolic KOH and the fatty acids extracted according to the procedure described by Han and Christie[25].

Thin layer chromatography

Thin layer chromatography (TLC) was performed as previously described[24] and confirmed total hydrolysis of the wax esters (Fig. S1, appendix). In brief, lipids extracts and standards (18-5 from Nu-check prep inc. MN, USA) were applied to high performance thin layer chromatography (HPTLC) plates (silica gel 60 matrix, Merck, Darmstadt, Germany), which were developed using a mobile phase consisting of heptane: diethyl ether: acetic acid (80:20:2). The TLC plates were dried and sprayed with 10% copper sulphate in 8% phosphoric acid. The major lipid species were visualized by heating for 10 min at 180 °C.

A separate TLC run, including several concentrations of a known fatty acid, was performed to determine the fatty acid concentration in the wax ester hydrolysate. The plate was scanned using Image Studio Lite v5.2.5 (LI-COR Biosciences - GmbH), and the data were subsequently used to establish a fatty acid standard curve, which in turn was used for determination of the fatty acid concentration in the wax ester hydrolysate. Since the hydrolysis of the wax ester was complete, and the wax ester is composed of one fatty alcohol and one fatty acid, we assumed equimolar concentrations of fatty acids and fatty alcohols in the hydrolysate.

Determination of cell viability

The effect of WE_H on palmitate-induced cell death was assessed by Live Cell Imaging (Zeiss Celldiscoverer, Carl Zeiss Microscopy, GmbH 07745 Jena, Germany). The number of live and dead cells were manually counted in images obtained at different time points throughout a 20 h incubation period. In addition, cell viability was analyzed by xCELLigence biosensor technology (xCELLigence® Biosensor Technology RTCA, ACEA Etterbeek, Belgium), which measures the strength of adhesion (in terms of electrical impedance) of cells to high-density gold electrode arrays printed on custom-designed E-plates (plates with gold microelectrodes fused to the bottom surface).

Baseline impedance (termed cell index, CI), was measured after 24 h preincubation of the cells (time zero), and measurements of impedance at 10 and 20 h were normalized to the zero-time value.

Immunofluorescence

H9c2 cells grown in Lab-Tek chambered coverglass (Thermo Scientific #155409) were fixed for 20 min with 4% formaldehyde in PHEM buffer, permeabilized with 0.3% Triton X-100 in PBS for 5 min and incubated with 3% goat serum in PBS for 60 min to block unspecific binding. The cells were then incubated overnight at 4° C with primary antibodies against CHOP (Cell Signaling #2895, diluted 1:400), LC3B (Sigma-Aldrich #L7543, diluted 1:1300), and p62/SQSTM1 (Progen #GP62-C, diluted 1:2000). After primary antibody incubation, cells were washed 6 x 5 min with PBS and then incubated with AlexaFluor-conjugated secondary antibodies (diluted 1:500) for 30 min at room temperature. Both primary and secondary antibodies were diluted in 1% goat serum in PBS. Finally, cells were washed 4 x 5 min with PBS, cell nuclei were stained with 1 µg/mL DAPI in PBS for 10 min, followed by two additional washes with PBS.

Fluorescence microscopy

The cells were imaged using an LSM880 confocal microscope or a CD7 widefield microscope (both systems Carl Zeiss Microscopy). Images were collected in ZEN software using a 40x NA1.2 W C-Apochromat objective for confocal microscopy, or a 50x NA1.2 W Plan-Apochromat objective for widefield microscopy. Images were recorded with a pixel size of 0.42 µm for confocal acquisition and 0.13 µm for widefield acquisition. In both cases, optimal excitation and emission settings for each fluorophore were determined using the Smart Setup function in ZEN. All fluorescence channels were recorded at non-saturating levels and acquisition settings were kept identical between all samples used for comparisons or quantifications. For scoring CHOP nuclear translocation and quantifying the per-cell amount of p62/SQSTM1 bodies between 15 and 20 confocal stacks (z step size = 1.00 µm,

z range = 5.00 µm) were collected at random positions in each well and used for subsequent image analysis.

Palmitate uptake in H9c2 cardiomyoblasts

In order to find out if the hydrolysate of Calanus oil-derived wax ester (WE_H) affected the uptake of fatty acids, we measured palmitate uptake in H9c2 cells over a 8 h period, using ^{3}H-labelled palmitate (0.2 µCi/mL) as tracer. Cells were collected at timed intervals and washed several times with cold PBS. Lipids were extracted by the method of Folch[26] and the radioactivity determined by liquid scintillation.

Real-time quantitative PCR

Cells were collected and immersed in RNAlater (Qiagen, Hilden, Germany), and total RNA was extracted according to the RNeasy plus mini kit Protocol (Qiagen Nordic, Norway). Quantification and purity of RNA was measured spectrophotometrically. RNA was reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher) and real-time PCR was performed in a LightCycler®96 System (Roche) with the cDNA and FastStart Essential DNA Green Master (Roche). Target gene expression levels were normalized to a stable expressed housekeeping gene (HMBS, GAPDH, SDHA, Cyclo). The stability of the housekeeping gene was determined by geNorm. CQ (quantification cycle)-value was defined as the cycle number at which the fluorescent signal was recorded above background level, defined by the LightCycle software[27].

Results

WE_H protects H9c2 cells from palmitate-induced cell death

Incubation of H9c2 cells in the presence of 100 μM palmitate (Palm) resulted in a time-dependent increase in cell death, as assessed by visual examination and scoring of live cell microscopy images (Fig. 1A). The first dead cells appeared after about 6-8 h incubation, while almost all cells were dead at 20 h incubation. The percentage of live palmitate-exposed cells after 10 and 20 h was approximately 65% and 25%, respectively, while the corresponding numbers for cells incubated in the presence of vehicle, were 99% and 97% (Fig. 1 B and D). Co-incubation of palmitate-exposed cells with hydrolysate of wax ester from Calanus oil (WE_H) resulted in a clear dose-dependent increase in cell viability. Thus, after 20 h cell viability was significantly improved in WE_H treated cells, even at very low concentrations (1 and 2 μM), while the highest dose (10 μM) almost completely rescued the cells from palmitate-induced death (Fig. 1 B and D).

To quantify the effect of palmitate more precisely with respect to cell viability, we used xCELLigence real-time cell analysis. Baseline cell index (CI) was measured following 24 h preincubation (time zero) and thereafter every 10 min over the next 20 h period. In vehicle-treated cells, CI increased continuously throughout the full 20 h period, while it started to decay after about 10 h in palmitate-exposed cells. In the same manner as with live cell imaging, we found that co-incubation with WE_H dose-dependently antagonized the palmitate-induced decline in CI, and the CI curve obtained with 10 μM WE_H was not significantly different from that of vehicle-treated cells (Fig. 1 C and E). Of note, measurements of palmitate uptake over time showed a drop in uptake after about 6-8 h (Fig. S2, appendix), as expected in light of the cell viability data shown in Fig. 1. Otherwise, palmitate uptake was not affected by co-incubation with WE_H, indicating that its observed protective effect is not linked to a reduction in palmitate uptake.

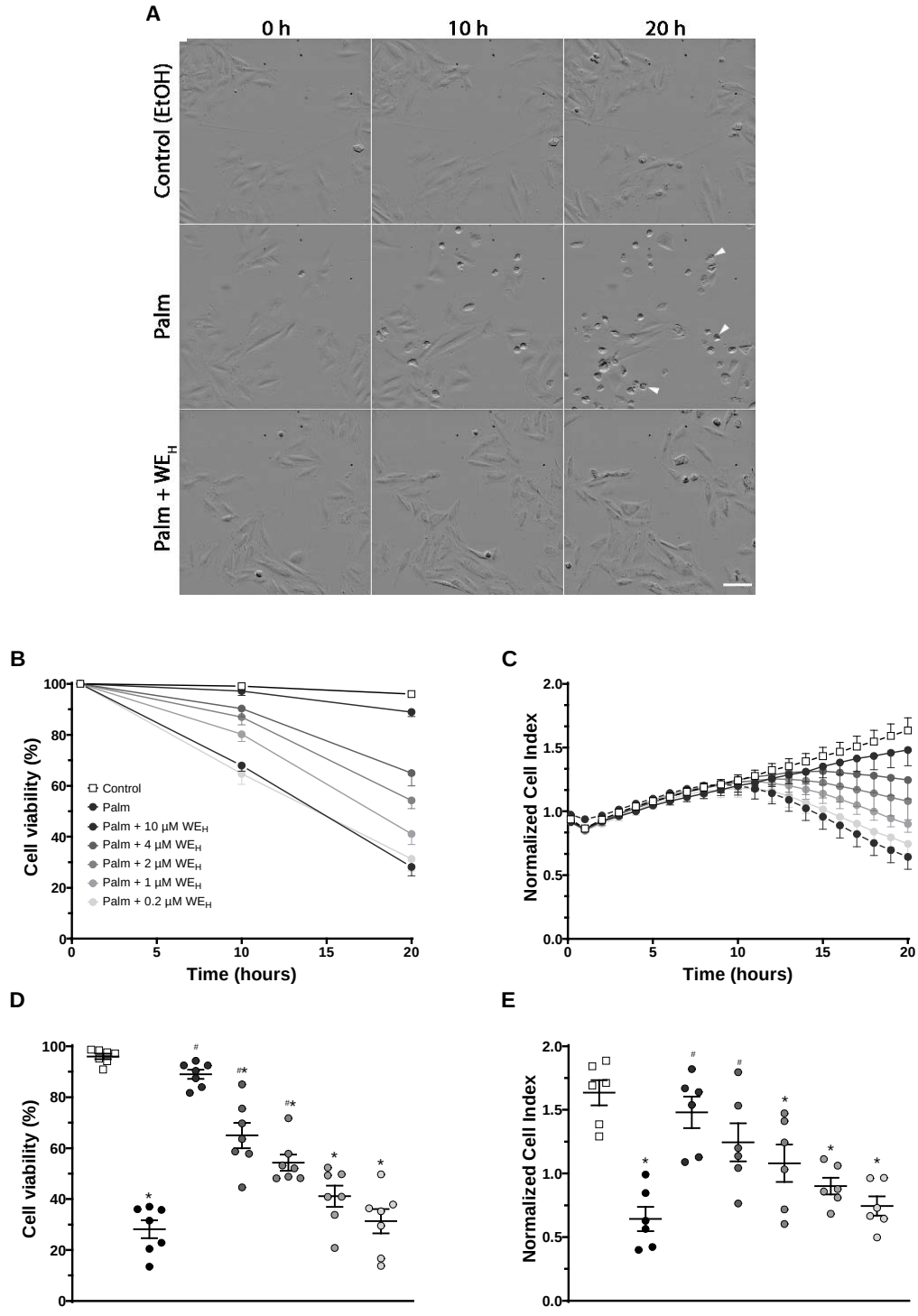

Figure 1. Effect of palmitate (Palm) and hydrolysed wax ester from Calanus oil (WE_H) on viability of palmitate-exposed H9c2 cells. The cells were incubated with 100 µM Palm and 0-10 µM WE_H for 20 hours. Ethanol (vehicle)-treated cells were included as controls**. A:** Representative live cell images showing palmitate-induced cell death (arrowheads indicate examples of dead cells). Scale bar 100 µm. **B and C**: Time courses of cell viability of palm-exposed H9c2 cells in the absence and presence of increasing concentrations of WE_H. Cell viability assessed as % live cells from the live cell images or Normalized Cell Index, using Live Cell Imaging and xCELLigence RTCA Biosensor Technology, respectively, respectively. **D and E**: Cell viability in individual experiments at the end of the 20 h incubation period. Data are mean ± SEM of n = 6 and 7 (Live Cell Imaging and xCELLigence, respectively). * $p<0.05$ vs. control; # $p<0.05$ vs palmitate.

WE_H reduces palmitate-induced ER stress

Palmitate has previously been shown to induce ER stress in H9c2 cells[6]. Thus, we investigated whether the observed protective effect of WE_H could be accompanied by reduced ER stress. To this end we conducted new live cell imaging experiments, confirming that 20 h palmitate exposure resulted in a marked reduction in cell viability, which was effectively prevented by co-incubation with 10 µM WE_H.

Salubrinal is a selective inhibitor of eIF2α dephosphorylation and protects cells from ER stress [28]. Interestingly, we found Salubrinal significantly reduced the palmitate-induced cell death (Fig. 2A), suggesting that survival of H9c2 cells after palmitate exposure is strongly affected by eIF2 phosphorylation status. To examine if WE_H could have a direct effect on ER-stress, we induced ER stress in control cells, using Tunicamycin (a protein glycosylation inhibitor)[28]. As expected, tunicamycin significantly reduced cell viability, however, co-incubation with WE_H did not alter tunicamycin-induced reduction in cell viability.

We also measured gene expression of the transcription factor CHOP, a marker of ER stress, and found a 7-fold increase following 6 h palmitate incubation (Fig. 2B). These data were corroborated by immunofluorescence microscopy of CHOP after 15 h, showing a clear increase in CHOP nuclear translocation after palmitate exposure (Fig. 2C). Taken together these results confirm that palmitate causes severe ER stress in H9c2 cells, which is in line with previous findings[6]. Importantly, co-incubation with 10 µM WE_H caused a marked reduction in both CHOP expression (Fig. 2B) and nuclear translocation (Fig. 2C). Incubation with WE_H alone had no effect on CHOP expression (Fig. 2B) or nuclear translocation (data not shown). Thus, these data clearly show that WE_H can counteract palmitate-induced up-regulation of CHOP in H9c2 cells.

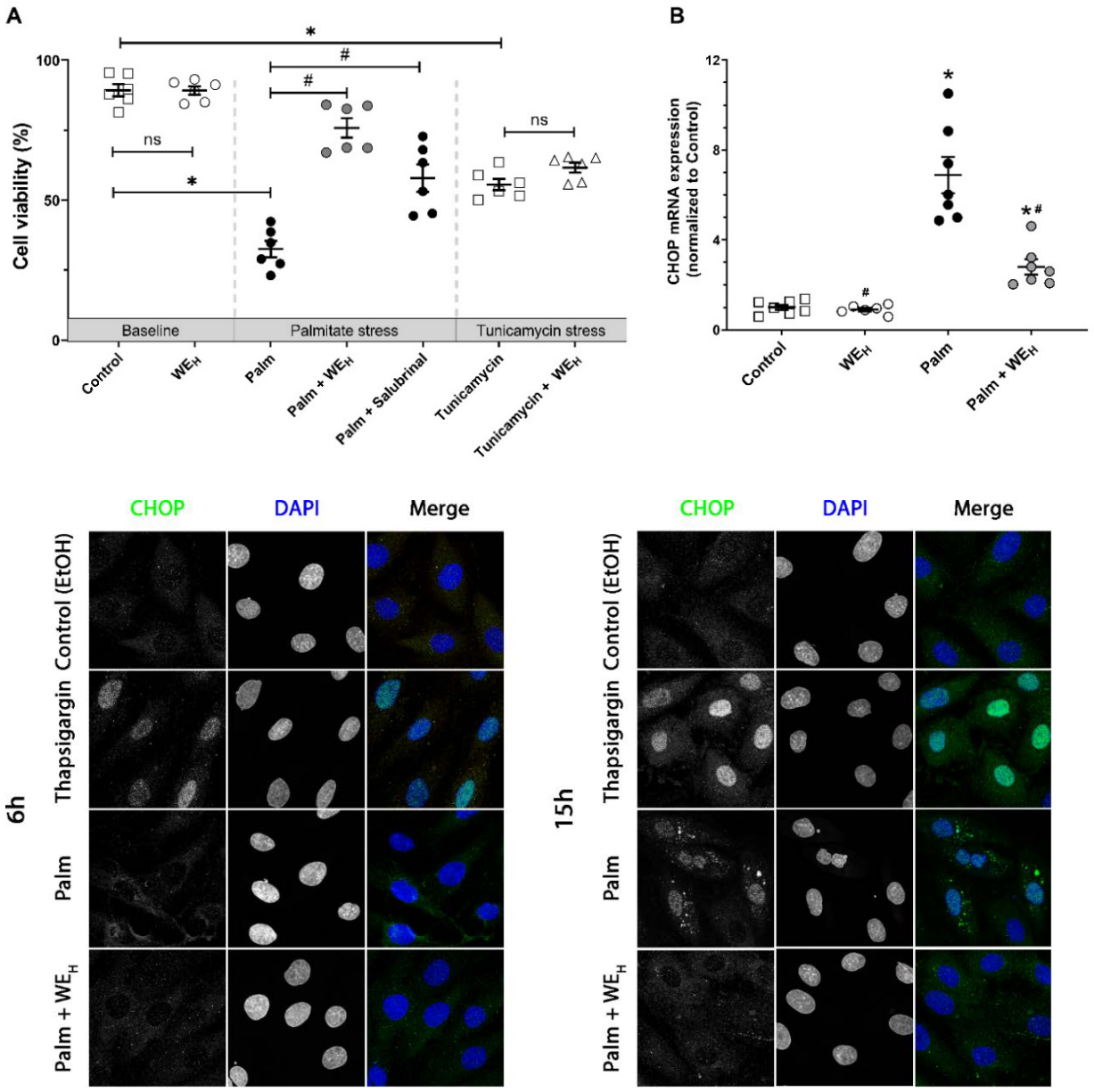

Figure 2. Effect of hydrolysed wax ester from Calanus oil (WE_H) and ER stress inhibition and induction in H9c2 cells. The cells were incubated with 100 µM palmitate (Palm) and 10 µM WE_H. Ethanol (vehicle)-treated cells were included as controls. **A**: Cell viability (% live cells assessed from Live Cell Images) following 20 h incubation with Palm and WE_H. Cells were also incubated in the presence of 60 µM salubrinal (ER stress inhibitor) or 2.5 µg/mL tunicamycin (ER stress inducer) as indicated. **B**: The mRNA expression of the CHOP gene in cells incubated with Palm and WE_H for 6 hours. **C:** The nuclear translocation of CHOP in cells incubated with Palm and WE_H for 6 and 15 hours. Cells were also incubated in the presence of 1 µM thapsigargin (ER stress inducer) as controls, respectively. * $p<0.05$ vs. control; # $p<0.05$ vs palmitate.

WE_H counteract the palmitate-induced impairment of autophagy flux

ER stress is dynamically interrelated with autophagy, and several reports have suggested that palmitate can impair autophagic flux. Thus, we also examined whether the observed protective effect of WE_H was accompanied by reduced palmitate-induced impairment of autophagic flux, using live cell imaging experiments for assessment of cell viability (Fig. 3A) and immunofluorescence studies with the autophagosome marker LC3B and the selective autophagy cargo receptor p62 (Fig. 3B). We first confirmed that H9c2 cells elicit a normal autophagy response with typical LC3B-positive autophagosomes appearing after acute aminco acid starvation (Hanks treatment) as well as an accumulation of both LC3B and p62 in cytosolic puncta after lysosomal inhibition with Bafilomycin A_1 (Fig. S3). In contrast, we found that palmitate exposure (6 h) induced numerous enlarged LC3B-positive vesicles (Fig. 3B, middle panel) and a dramatic increase in p62-positive aggregates/inclusions. It should be noted that these structures appeared already within two hours of treatment and were present at all later timepoints.

Importantly, in cells co-treated with palmitate and WE_H the presence of LC3B vesicles and p62 aggregates/inclusions was greatly diminished (Fig. 3B, lower panel), which suggests that WE_H can counteract this palmitate-induced impairment of the autophagic flux. Therefore, we tested if activation of autophagy could increase viability in palmitate-treated cells. Inducing autophagy with rapamycin did not counteract the palmitate-induced cell death. In addition, we examined whether WE_H could ameliorate the cell death induced by inhibiting endogenous autophagy using LY294002. As shown in Fig. 3A, WE_H did not protect cell death following chemical inhibition of autophagy. Taken together, these results suggest that autophagy is impaired by palmitate-induced stress in H9c2 cells, but chemical activation of autophagy does not alleviate palmitate-induced cell death. Moreover, the protective effect of WE_H on cell viability is likely not due to a direct effect on the autophagic flux.

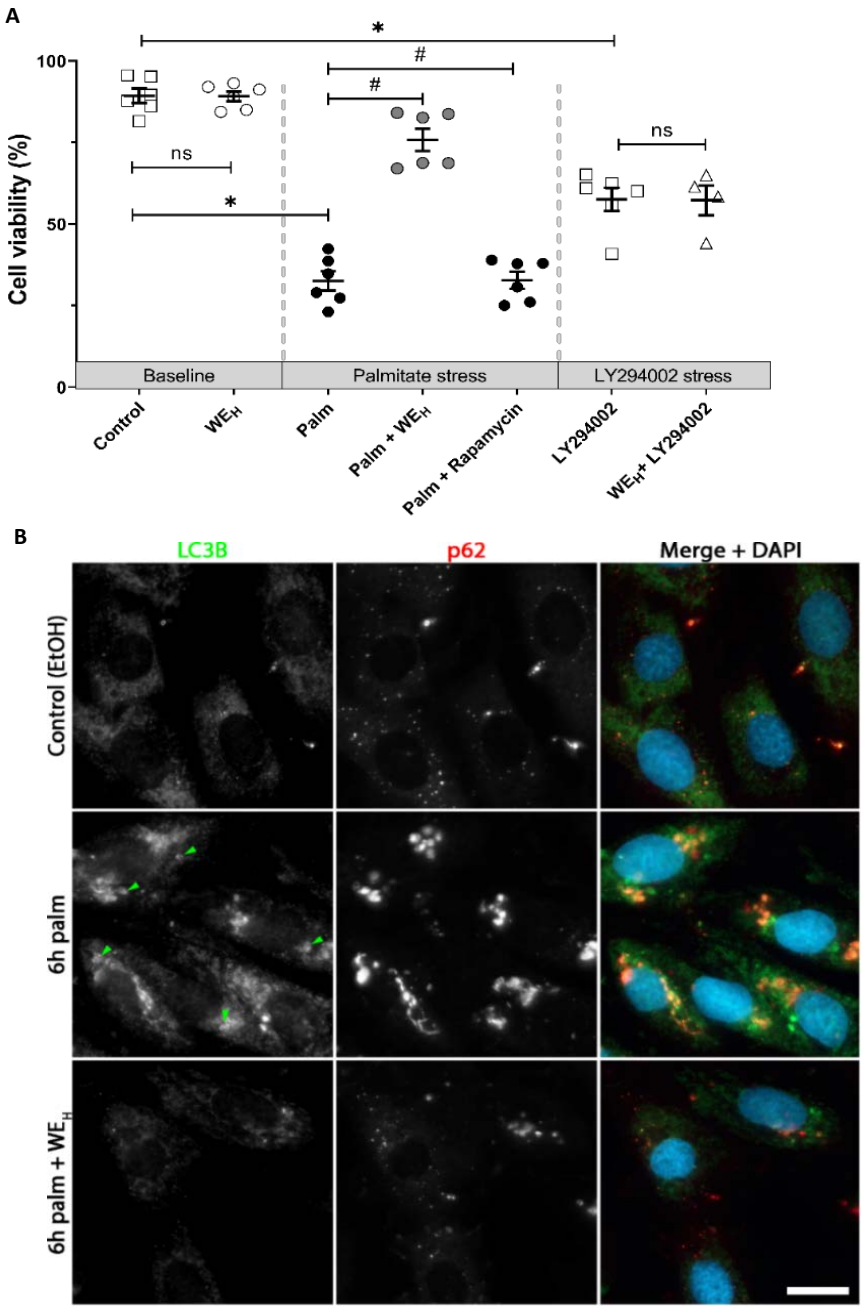

Figure 3. Effect of palmitate (Palm) and hydrolysed wax ester from Calanus oil (WE_H) on cell viability and autophagy in H9c2 cells. The cells were incubated with 100 µM Palm and 10 µM WE_H. Ethanol (vehicle)-treated cells were included as controls. **A**: Cell viability (% live cells assessed from Live Cell Images) following 20 h incubation with Palm and WE_H. Cells were also incubated in the presence of 1µM rapamycine or 20 µM LY294002 as indicated. **B:** The abundance and localization of LC3B and p62 (autophagy markers) in cells incubation with Palm and WE_H for 6 h. The cells were imaged by confocal microscopy. Green arrowheads point to enlarged LC3B vesicles. Scale bar 20 µm.

Discussion

In a recent study we found that dietary supplementation with Calanus® oil is cardioprotective, in the sense that it attenuates ischemic damage in perfused hearts from diet-induced obese mice[23]. This oil has a unique chemical composition, containing 80-85% lipids in the form of wax esters. In the present study, we show that hydrolysed wax ester from Calanus® oil (WE_H) effectively and dose-dependently protects H9c2 cells from palmitate-induced cell death.

Animal experiments have reported cardioprotective effects of dietary fish oil supplements by reporting improved functional recovery or decreased infarct size following ischemia [29, 30]. A protective effect of marine polyunsaturated fatty acids (PUFA), such as EPA and/or DHA, was also reported in palmitate-exposed H9c2 cells. However, in these experiments, the concentrations of PUFA were considerably higher than those used in the current study; Cetrullo et al.[31] reported that 80 µM EPA significantly increased survival of H9c2 cells incubated with 500 µM palmitate. The same group also found that the combination of EPA and DHA (60 µM each) protected the cells from the palmitate stress[32]. In contrast, we demonstrate in this study that 10 µM WE_H nearly completely abolished the palmitate-induced cell death. It is not obvious why the wax ester hydrolysate afforded protection at concentrations below 10 µM, but the combination of several PUFAs, including a relatively high concentration of stearidonic acid (SDA) as well as monounsaturated fatty acids, could probably explain its efficacy. In addition, fatty acids and fatty alcohols were present at the same molar concentrations in the wax ester hydrolysate, and although the beneficial effects of marine oils are normally attributed to their content of PUFA, the presence of fatty alcohols could also contribute to the potency of the hydrolysate[33].

PUFA play a pivotal role for regulating the biophysical properties of cellular membranes[34], and human studies suggest that replacement of saturated fat in the diet by PUFA may contribute to lower uptake of lipids in skeletal muscle[35]. We therefore examined whether the protective effect of WE_H

could be explained in terms of reduced lipotoxicity due to reduced palmitate uptake. However, the results showed that palmitate uptake was not affected by the presence of $WE_{H.}$

Endoplasmatic reticulum (ER) stress and autophagy are two main processes affected during palmitate-induced lipotoxicity [6, 8]. Accordingly, we observed that palmitate exposure resulted in a significant increase in the expression and nuclear translocation of CHOP (an established marker of ER stress), and that inhibition of ER stress with salubrinal significantly improved viability in palmitate-exposed cells. Importantly, co-incubation with WE_H markedly ameliorated both the palmitate-induced increase in CHOP expression and translocation to the nuclei. These data might suggest that prevention or reduction of ER stress, at least in part, accounts for the cardioprotective effect of $WE_{H.}$ On the other hand, WE_H did not inhibit tunicamycin-induced cell death, which indicates that its cardioprotective effect could be related to amelioration of the palmitate-mediated stress on the cells.

The present study supports previous reports, which show that palmitate induces LC3B-positive vesicles in H9c2 cells[6, 17]. This increase was accompanied by p62-positive aggregates/inclusions, as also shown by Jaishy et al.[17]. However, Park et al.[6] noted a decrease in p62 expression which could probably be related to a less severe palmitate stress avoiding impairment of autophagic flux. Finally, these structures were apparent within two hours of treatment and did not diminish over time, suggesting an impairment of autophagic flux already at an early stage of the process.

Of particular interest, however, was the observation that the presence of LC3B vesicles and p62 aggregates/inclusions were absent in palmitate-exposed cells co-treated with WE_H. In our hands, however, activation of autophagy (by co-treatment with rapamycin) failed to affect viability of palmitate-treated H9c2, indicating that pharmacological activation of autophagy alone is not sufficient to alleviate cellular toxicity caused by palmitate. Taken together, these data suggest that

palmitate impairs autophagic flux, and that WE_H prevents the formation of palmitate-induced aggregates/inclusions, most likely due to reduction of the palmitate-induced stress.

In conclusion, this study demonstrates for the first time that the constituents of the wax ester from Calanus oil, namely a unique composition of fatty acids and fatty alcohols, has a direct protective effect on cardiac cells during nutritional stress. Low concentrations of this hydrolysate protect H9c2 cardiomyoblasts from palmitate-induced lipotoxicity by inhibiting or preventing ER stress and impairment of autophagic flux. Further studies are, however, needed to fully reveal the mechanisms involved.

Acknowledgements

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Supplemented data

Figure S1. Thin layer chromatography showing the various lipid classes in crude Calanus oil in line 1; purified wax ester in lane 2; hydrolysed wax ester (i.e. fatty acids and fatty alcohols) in lane 3; lipid standard (18:5 from Nu-check prep inc.) in lane 4.

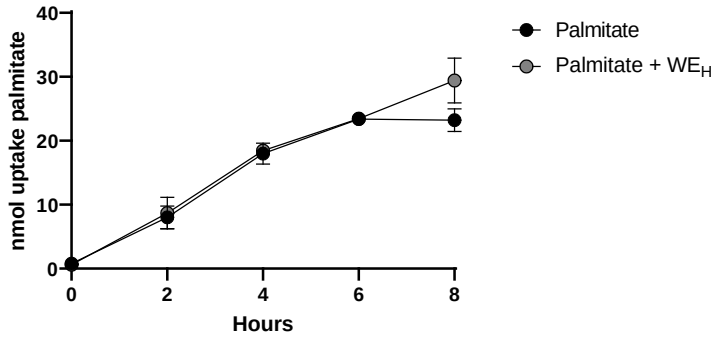

Figure S2. Palmitate uptake in H9c2 cells incubated with palmitate with or without hydrolysed wax ester (WE_H). The cells were incubated with 100 µM palmitate (Palm) and 10 µM WE_H over time. Palmitate uptake was measured during incubation in 6-well plates, using ^3H labeled palmitate as tracer. The data is expressed as nmol uptake per well. Data are mean ± SEM of two experiments.

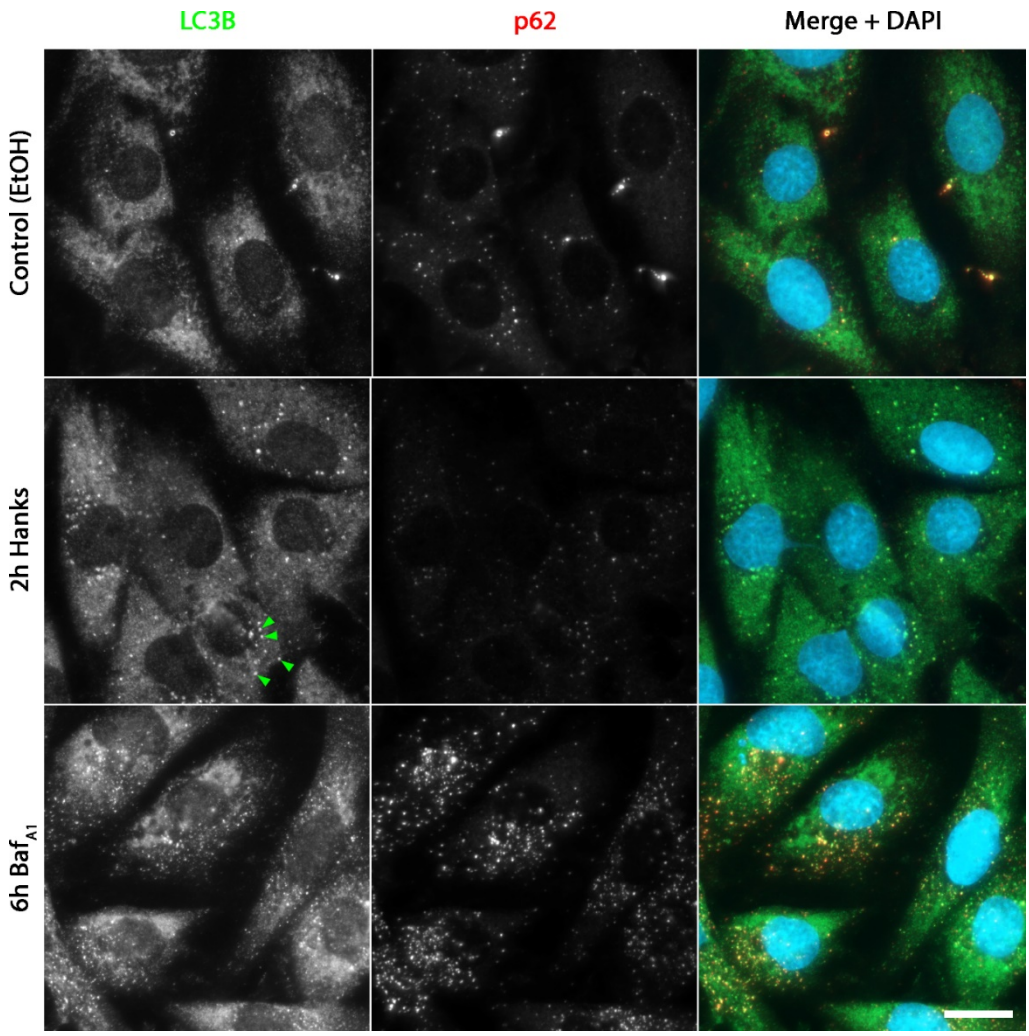

Figure S3. Effect of autophagy activation and autophagic flux inhibition on localization and abundance of LC3B and p62. Immunofluorescence microscopy of H9c2 cells acute amino acid starvation (2h Hanks) or lysosomal inhibition (6h BafA1). Ethanol-treated cells were included as a control. Scale bar 20 µm.

Paper III:

Obesity-induced alterations in the gut microbiome in female mice fed a high-fat diet are antagonized by dietary supplementation with a novel, wax ester– rich, marine oil

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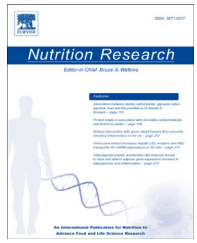

Obesity-induced alterations in the gut microbiome in female mice fed a high-fat diet are antagonized by dietary supplementation with a novel, wax ester–rich, marine oil

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ABSTRACT

Dietary supplementation with calanus oil, a novel wax ester–rich marine oil, has been shown to reduce adiposity in high-fat diet (HFD)–induced obese mice. Current evidence suggests that obesity and its comorbidities are intrinsically linked with unfavorable changes in the intestinal microbiome. Thus, in line with its antiobesity effect, we hypothesized that dietary supplementation with calanus oil should counteract the obesity-related deleterious changes in the gut microbiota. Seven-week-old female C57bl/6J mice received an HFD for 12 weeks to induce obesity followed by 8-week supplementation with 2% calanus oil. For comparative reasons, another group of mice was treated with exenatide, an antiobesogenic glucagon-like peptide-1 receptor agonist. Mice fed normal chow diet or nonsupplemented HFD for 20 weeks served as lean and obese controls, respectively. 16S rRNA gene sequencing was performed on fecal samples from the colon. HFD increased the abundance of the *Lactococcus* and *Leuconostoc* genera relative to normal chow diet, whereas abundances of *Allobaculum* and *Oscillospira* were decreased. Supplementation with calanus oil led to an apparent overrepresentation of *Lactobacillus* and *Streptococcus* and underrepresentation of *Bilophila*. Exenatide prevented the HFD-induced increase in *Lactococcus* and caused a decrease in the abundance of *Streptococcus* compared to the HFD group. Thus, HFD altered the gut microbiota composition in an unhealthy direction by increasing the abundance of proinflammatory genera while reducing those considered health-promoting. These obesity-induced changes were antagonized by both calanus oil and exenatide.

Abbreviations: Cal, calanus oil; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; Ex, exenatide; GLP-1, glucagon-like peptide-1; HFD, high-fat diet; HFD + Cal, HFD supplemented with 2% (w/w) Calanus Oil; HFD + Ex, HFD plus treatment with exenatide; NCD, normal chow diet; PUFA, polyunsaturated fatty acids; SDA, stearidonic acid; WAT, white adipose tissue.

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1. Introduction

The role of gut microbiota in human health has received significant interest in recent years. Alterations in the composition of the gut microbiome have been associated with obesity and metabolic disorders, such as insulin resistance and type 2 diabetes mellitus, thereby increasing the risk for cardiometabolic disease [1-5]. The causal relationship between alterations in the gut microbiota and disease development is, however, still unclear, and it is not fully understood if changes in the microbial composition occur before or after disease onset [6]. However, it is known that diet is an important driver behind changes in the gut microbiota [7-9].

Humans have low ability to synthesize the long-chain omega-3 polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA). It is therefore necessary to obtain these essential fatty acids via the diet. Studies describing the impact of EPA and DHA on the gut microbiota in humans are relatively sparse. Rajkumar et al. [10] were the first to study the impact of EPA and DHA on human gut microbiota but failed to detect any major effects on its composition. Later studies providing omega-3 PUFA supplements to both healthy and obese individuals revealed similar changes in both groups, such as a decrease in *Faecalibacterium*, often associated with an increase in the Bacteroidetes and butyrate-producing bacteria of the Lachnospiraceae family [11-13]. Animal studies have also reported alterations in gut microbiota following dietary supplementation with marine oils, and interestingly, these alterations in the microbiome were associated with reductions in body weight gain and white adipose tissue (WAT) inflammation [14] as well as a reduced adiposity index [15].

Calanus oil is a novel marine oil extracted from the copepod *Calanus finmarchicus*. The oil consists mainly of wax esters, a lipid class where fatty acids are esterified to long-chain fatty alcohols [16]. This novel marine oil is, compared to other marine oils, relatively low in EPA and DHA (6% and 4%, respectively) but relatively rich in stearidonic acid (18:4n-3, SDA, 7%), the precursor of EPA and DHA [17,18]. In addition, about 11% of the fatty acids present in the oil are (long-chain) monounsaturated fatty acids. The main fatty alcohols are represented by the equivalents of the dominant monounsaturated fatty acids, namely, eicosenol (20:1n-9) and docosenol (22:1n-11) [18].

Digestion and utilization of wax esters are poorly understood [19], although it is evident that humans are able, to some extent, to hydrolyze waxes and absorb the liberated fatty acids and alcohols [20]. Furthermore, previous studies in mice have shown that the wax esters are hydrolyzed and absorbed in the intestine and that the wax ester–derived fatty acids are incorporated in liver and WAT [18]. Dietary lipids, such as triacylglycerol and phospholipids, are quickly digested and absorbed in the upper parts of the gastrointestinal tract and will normally not reach the lower part [21]. Wax esters are hydrolyzed by a bile salt–dependent pancreatic carboxyl esterase [20]. They are relatively hydrophobic and therefore more difficult to emulsify, and as a result, wax esters may exhibit a longer retention time [22,23], allowing release of the liberated fatty acid and fatty alcohols in the distal part of the intestine.

The mouse model of diet-induced obesity shares many of the same characteristics as human obesity [24] and has become an important tool for understanding the pathological mechanisms involved in obesity-related diseases, such as insulin resistance, oxidative stress, and liver steatosis [25]. In particular, the development of a low-grade inflammatory state in obese adipose tissue and altered adipose tissue metabolism with increased release of fatty acids are believed to play a central role in the development of obesity-related pathology.

Previous studies reported that dietary supplementation with a small amount (2%) of oil from *C finmarchicus* significantly reduced intra-abdominal and ectopic fat deposition in male mice during high-fat feeding [26,27]. Because obesity is characterized by unfavorable alterations in the composition and function of the gut microbiome [4], we hypothesized that reduced obesity following intake of calanus oil could prevent or antagonize such alterations of the bacterial composition in the gut. To test this hypothesis, normal mice were made obese during a 12-week period on high-fat diet (HFD). This was followed by 8 weeks of feeding on HFD with or without 2% calanus oil. For comparative reasons, we also included a group of HFD mice that were treated with the antidiabetic compound exenatide. This glucagon-like peptide-1 (GLP-1) receptor agonist also reduces fat deposition in mice during high-fat feeding [28].

2. Methods and materials

2.1. Study design and animals

All animal experiments were approved by the local authority of the National Animal Research Authority in Norway (FOTS id 8430). All mice were treated according to the guidelines on accommodation and care of animals formulated by the European Convention for the Protection of Vertebrate Animals for Experimental and Other Scientific Purposes. The animals were housed at 21°C, 3 animals per cage, under a reversed light/dark cycle (12-hour dark/12-hour light). The animals had ad libitum access to food and drinking water, and body weight and food intake were recorded once a week.

In this study, 4 groups of 5- to 6-week-old C57Bl/6J female mice (Charles River, Sulzfeld, Germany) were studied (5 mice per group). The first group served as the lean control group and was fed a normal chow diet (NCD) containing 10% of energy from fat (NCD, no. 58Y2, Test Diet; IPS Limited, Richmond, Indiana, USA) for 20 weeks. The other groups were diet-induced obese by feeding a lard-based high-fat diet containing 46% of energy from fat (HFD, no. 58V8, Test Diet; IPS Limited). The second group received the HFD for 20 weeks. The third and fourth groups received the HFD for 12 weeks followed by 8 weeks of treatment. The third group received HFD supplemented with 2% (wt/wt) commercial Calanus Oil, supplied by Calanus AS, Tromsø, Norway (HFD + Cal). The addition of the oil was compensated for by removal of 2 g lard/100 g diet, making the total fat and energy content of the HFD and the HFD + Cal similar. See Table 1 for a list of ingredients of the different diets and Table 2 for a list of fatty acid composition of Calanus Oil, the HFD, and the HFD + Cal. The nutritional profiles of the diets are shown in the supplementary data. The fourth group was fed HFD and treated with exenatide, 10 μg/kg/d, via miniosmotic pumps implanted subcutaneously at the back of the animals (HFD + Ex). After

Table 1 – Ingredient composition in g/kg of the 3 experimental diets

Ingredients	NCD	HFD	HFD + Calanus
Sucrose	331.29	200.92	200.92
Dextrin	298.56	84.83	84.83
Casein, vitamin tested	189.56	233.06	233.06
Powdered cellulose	47.39	58.27	58.27
Maltodextrin	33.17	116.53	116.53
Soybean oil	23.7	29.13	29.13
Lard	18.96	206.84	186.84
Calanus Oil-841	0	0	20
Potassium citrate, tribasic monohydrate	15.64	19.23	19.23
Calcium phosphate	12.32	15.15	15.15
DIO mineral mix	9.48	11.65	11.65
AIN-76A vitamin mix	9.48	11.65	11.65
Calcium carbonate	5.21	6.41	6.41
L-Cystine	2.84	3.5	3.5
Choline bitartrate	1.9	2.33	2.33
FD&C yellow no. 5	0.5	0	0
FD&C red 40 lake	0	0.5	0
Green dye	0	0	0.5
Total g/kg	1000	1000	1000
Total energy in kcal/g (kJ/g)	3.76 (15.73)	4.6 (19.25)	4.6 (19.25)

NCD, normal control diet (TestDiet 58Y2 with 10% energy from fat); HFD, high-fat diet (TestDiet 58V8 with 45% energy from fat); HFD + Cal, high-fat diet with 2% Calanus Oil (TestDiet 58V8 with 2% Calanus Oil). See supplementary materials for details.

implantation of the miniosmotic pumps, the mice were single housed to avoid animal interaction, which could damage the surgical wound. Mice that did not receive pumps were also single housed to control for possible effects of single housing.

Table 2 – Fatty acid composition (mg/g lipid) of Calanus Oil and experimental diets

Fatty acids	Calanus Oil	HFD	HFD + Cal
14:0	64.42	10.41	11.95
16:0	45.05	173.65	149.83
18:0	2.42	106.63	92.16
20:0	0.40	1.70	1.37
16:1*n-7*	17.17	10.95	9.98
18:1*n-7*	1.53	15.84	13.52
18:1*n-9*	15.54	243.82	208.42
20:1*n-9*	24.01	4.74	5.64
20:1*n-11*	3.90	ND	ND
22:1*n-9*	2.63	ND	ND
22:1*n-11*	43.33	ND	2.20
24:1*n-9*	2.81	ND	ND
18:2*n-6*	6.64	133.04	116.06
18:3*n-3*	13.72	12.49	11.67
18:4*n-3*	69.58	ND	4.54
20:2*n-6*	0.71	3.15	2.69
20:4*n-6*	1.39	0.48	1.15
20:5*n-3*	54.73	ND	3.35
22:5*n-3*	2.96	ND	ND
22:6*n-3*	39.35	ND	2.81

Modified from Pedersen et al. [18]. ND, not detected.

At the end of the feeding experiment, the mice were killed with an overdose of pentobarbital (100 mg/kg, 300 μL intraperitoneally). Organs were carefully dissected out and stored at –80 °C until further analysis. Feces samples were collected from the colon for microbiota analysis, after which the gastrointestinal tract was rinsed with saline before freezing.

2.2. *Fatty acid analysis of the colon wall and abdominal fat*

Fatty acids were methylated by dissolving 60 mg tissue sample in 2 mol/L HCl in methanol with 0.05% BHT and heating for 2 hours at 100 °C, and the fatty acid composition was determined by gas chromatography using an Agilent 6890N (Agilent Technologies, Santa Clara, CA, USA) gas chromatograph with a Varian CP7419 capillary column (Varian Inc, Palo Alto, CA, USA). The content of the individual fatty acids in the samples was expressed in percent of the total fatty acid content.

2.3. *Quantitative real-time polymerase chain reaction*

The expression of mRNA of genes related to inflammation and fat metabolism in the colon and omental WAT was determined using quantitative reversed real-time polymerase chain reaction (PCR). Samples of the intestinal wall (colon, n = 6-8 per group) were immersed in RNA later (Qiagen, Hilden, Germany), and WAT samples (n = 6-8 per group) were immersed in Allprotect Tissue Reagent (Qiagen, Hilden, Germany) and thawed overnight at 4°C. Thirty to 40 mg colon tissue and 50-100 mg WAT tissue were used for RNA extraction according to the Rneasy Lipid Tissue kit Protocol (Qiagen). RNA concentrations were measured spectrophotometrically (Nanodrop 2000, Thermo Fisher Scientific, Waltham, MA, USA) and stored at –80 °C before use for cDNA. cDNA was subsequently made from 2 μg total RNA according to High Capacity cDNA reverse transcriptase kit (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was stored at –20 °C until real-time PCR (quantitative PCR) was performed in a Roche LightCycler 96 using a 1:4 dilution of the cDNA and the fast-start essential DNA green master (Roche, Basel, Switzerland). Five housekeeping genes were analyzed to normalize the expression of the target genes to the geometric mean of the 2 best housekeeping genes, which were selected on the basis of the average expression stability values determined with geNorm. For quantification of the gene expression in the colon, the housekeeping genes *sdha* (succinate dehydrogenase complex subunit A) and *hprt* (hypoxanthine-guanine phosphoribosyl transferase) were used, and for the WAT, *sdha* and *gapdh* (glyceraldehyde-3-phosphate dehydrogenase) were used. Forward and reverse primers of the target genes analyzed in the colon and omental fat are shown in Table 3.

2.4. *Bacterial DNA isolation*

Total microbial DNA was isolated from feces samples with a QIAamp PowerFecal DNA kit (Qiagen) according to the manufacturer's protocol, with the following exceptions. The disintegration of the cells was done with a FastPrep device at a vortexing speed of 6.5 m/s for 30 seconds followed by

Table 3 – Forward and reverse primers of the target genes analyzed in the intestinal (colon) wall and omental fat

Gene	Accession number	Primer	Melting temperature (°C)	Product size
SDHA	NM_023281	F: TGT TCA GTT CCA CCC CAC A R: CAC GAC ACC CTT CTG TGA TG	59.0 59.0	62 bp
GAPDH	NM_001289726.1	F: TCA CCA CCA TGG AGA AGG C R: GCT AAG CAG TTG GTG GTG CA	59.3 61.2	169 bp
HPRT	NM_013556.2	F: TCC TCC TCA GAC CGC TTT T R: CCT GGT TCA TCA TCG CTA ATC	58.2 56.6	90 bp
NFkB	NM_008689.2	F: ATG GCA GAC GAT GAT CCC TAC R: CGG AAT CGA AAT CCC CTC TGT T	59.4 60.4	167 bp
GPR 120	NM_181748.2	F: GTG CCG GGA CTG GTC ATT GTG R: TTG TTG GGA CAC TCG GAT CTG G	63.8 62.3	123 bp
EMR1	NM001355722.1	F: TTG TAC GTG CAA CTC AGG ACT R: GAT CCC AGA GTG TTGATG CAA	59.6 58.3	144 bp
CD36	NM_001159558.1	F: TTG TAC CTA TAC TGT GGC TAA ATG AGA R: CTT GTG TTT TGA ACA TTT CTG CTT	59.7 57.4	72 bp
MCP1	NM_011333.3	F: TTA AAA ACC TGG ATC GGA ACC R: GCA TTA GCT TCA GAT TTA CGG)	56.2 55.1	121 bp
TLR4	NM_ 021297.3	F: TTC TTC TCC TGC CTG ACA CC R: CTT TGC TGA GTT TCT GAT CCA T	59.3 56.7	94 bp
Adiponectin	NM_009605.5	F: CCT GGC CAC AAT GGC ACA CCA R: GTG ACG CGG GTC TCC AGC C	65.8 65.5	233 bp
PPARα	NM_011144.6	F: ACG ATG CTG TCC TCC TTG ATG R: GTG TGA TAA AGC CAT TGC CGT	60.1 59.5	67 bp
PPARδ	NM_011145.3	F: GCT GCT GCA GAA GAT GGC A R: CAC TGC ATC ATC TGG GCA TG	61.0 59.3	63 bp
PPARγ	NM_001127330.2	F: CCA TTC TGG CCC ACC AAC R: AAT GCG AGT GGT CTT CCA TCA	58.3 59.7	67 bp
FIAF	NM_020581.2	F: GCC ACC AAT GTT TCC CCC AAT G R: TAC CAA ACC ACC AGC CAC CAG AGA	62.6 65.7	118 bp
IL-1β	NM_008361.4	F: TGT AAT GAA AGA CGG CAC ACC R: TCT TCT TTG GGT ATT GCT TGG	58.9 56.1	68 bp
MUC2	NM_023566.4	F: ATG CCC ACC TCC TCA AAG AC R: GTA GTT TCC GTT GGA ACA GTG AA	59.7 59.1	101 bp
GLP2R	NM_ 175 681.3	F: TCA TCT CCC TCT TCT TGG CTC TTA C R: TCT GAC AGA TAT GAC ATC CAT CCAC	61.6 60.0	196 bp
IL-18	NM_008360.2	F: CAT GTA CAA AGA CAG TGA AGT AAG AGG R: TTT CAG GTG GAT CCA TTT CC	59.9 55.3	122 bp
ZO-1	NM_009386.2	F: GAG CGG GCT ACC TTA CTG AAC R: GTC ATC TCT TTC CGA GGC ATT AG	60.5 59.0	75 bp
IFNγ	NM_008337.4	F: TTG GCT TTG CAG CTC TTC CT R: TGA CTG TGC CGT GGC AGT A	60.2 61.5	58 bp
Occludin	NM_008756.2	F: TTG AAA GTC CAC CTC CTT ACA GA R: CCG GAT AAA AAG AGT ACG CTG G	59.0 59.1	129 bp

incubation at 70 °C for 5 minutes. The vortexing and incubation were repeated once. The elution was done with 100 μL of elution buffer and stored at –20 °C until used.

2.5. *16S rDNA amplification*

Twenty nanograms of DNA was used to prepare PCR amplicons of the V4-V5 region of 16S rRNA according to Fliegerova et al. [29]. The mixture contained OneTaq DNA Polymerase (New England Biolabs, Ipswich, Massachusetts, USA), and the thermal profile consisted of initial denaturation for 5 minutes at 95 °C followed by 35 cycles of 30 seconds at 95 °C, 30 seconds at 57 °C, and 30 seconds at 72 °C and a final elongation for 5 minutes at 72 °C. The PCR amplicons were checked by electrophoresis in 1.5% agarose (25 minutes at 90 V), purified with a QIAquick PCR Purification Kit (QIAGEN) according to the protocol, and quantified by Nanodrop OneC.

2.6. *Next-generation sequencing*

The obtained PCR products were used to prepare amplicon libraries for diversity analyses by a next-generation sequencing approach on a Personal Genome Machine (Life Technologies, Carlsbad, California, USA) according to Milani et al. [30]. Two hundred nanograms of DNA from each sample was used to prepare sequencing libraries with a NEBNext Fast DNA Library Prep Set kit (New England Biolabs) according to the manufacturer's protocol. Ion Xpress Barcode adapters (Thermo Fisher Scientific) were used to label each sample. The obtained libraries were used to prepare a sequencing template by using an Ion PGMTM Hi-QTM View OT2 Kit (Thermo Fisher Scientific). The template was then sequenced on an Ion 316TM Chip Kit v2 (Thermo Fisher Scientific) by using an Ion PGMTM Hi-QTM View Sequencing kit (Thermo Fisher Scientific). Two independent sequencing runs were performed.

2.7. Next-generation sequencing data analyses

The sequences were obtained in the form of the FASTQ format and further processed by the QIIME2 analyses pipeline [31]. The sequences were demultiplexed and dereplicated using the versatile and open-source tool VSEARCH [32]. The bioinformatics software PICRUSt v 1.1.3 was applied for both the taxonomical and functional analyses [33]. The sequences were clustered and identified by performing closed-reference operational taxonomic unit (OTU) picking selecting a cutoff at 90% sequence identity against the Greengene reference OTUs (gg_13_5_otus). The resulting OTU table was normalized using the *normalize_by_copy_number.py* script. Functional trait abundance was predicted using the *predict_metagenomes.py* script. The predictions were collapsed into Kyoto Encyclopedia of Genes and Genomes pathways level 3 using the *categorize_by_function.py* script, and the resulting abundance table was imported in the bioinformatics software STAMP for statistical analysis [34].

Diversity index analysis and unweighted UniFrac distance metrics analysis [35] were carried out by exporting the QIIME2 biom file to MicrobiomeAnalyst. The Web site microbiomeanalyst.ca was used for visualization of the α diversity index and the heat map. The absolute and relative abundance tables were exported and used in STAMP for statistical analysis of the taxonomy data, in GraphPad prism 8.2.1 for the (stacked) bar graphs, and in MEGAN Community Edition (version 6.15.2) for visualization of the principal component analysis.

2.8. Statistical analyses

Data are presented as the means with their standard errors. Statistical analysis of the functional and taxonomic data was done using STAMP [34]. Noncorrected 2-sided Student *t* test between the HFD group and the other three groups was done to check for significant differences vs the HFD group (n = 5 for each group). Mean differences were considered significant at $P < .05$. Statistical analysis on the gene sequencing was done in GraphPad prism 8.2.1. Differences between treatment groups were assessed by 1-way analysis of variance followed by Dunnet post hoc test where the groups were compared to the HFD group (n = 6-8 for each group). Sample sizes were determined based on availability of the samples because this study was ran in parallel to another study using the same mice [36].

3. Results

3.1. Body weight gain and fatty acid composition of the diets, and abdominal fat and colon wall

The present microbiota data were obtained on a subgroup of mice (n = 5) used in a recent publication by Jansen et al. [36]. Details about body weight development, weight of adipose tissue, and food intake are given in that publication. Here it is just pointed out that supplementation of the HFD with oil from *C finmarchicus* for the last 8 weeks did not affect body weight gain (Fig. 1), although it did result in a lower adiposity index (total weight of intra-abdominal fat depots) relative to the nonsupplemented HFD group. Treatment with exenatide, however, led to both lower weight gain (Fig. 1) and lower adiposity index. Of note, both treatments recovered the capacity of the heart to oxidize glucose, which was otherwise impaired in response to high-fat feeding [36], demonstrating that both the wax ester–rich oil and exenatide recovered metabolic flexibility of hearts from diet-induced obese mice. There were no differences in food intake between the diet groups during the treatment period [36]. The inclusion of 2% calanus oil in the HFD resulted in clearly detectable amounts of SDA, EPA, and DHA in this diet (Table 2). Analysis of the fatty acid composition of abdominal fat showed a statistically significant increase in the content of n-3 PUFA (SDA, DHA, and EPA), whereas the content of arachidonic acid (n-6 PUFA) was significantly reduced (Table 4). In the colon wall, the content of SDA was significantly increased ($P < .05$) in the HFD + Cal group relative to that of the HFD group (0.89% ± 0.23% vs 0.63% ± 0.07%), but for the other PUFA, the variability was too high to reach firm conclusions (data not shown).

3.2. Microbiota composition of feces samples from colon

The average sequence count over all 20 samples was 6761 sequence reads per sample. The average species diversity, or α diversity, within the 4 diet groups was measured using the Shannon Phylogenetic Diversity index on the OTU level (Fig. 2). No statistically significant differences were observed between the groups ($P = .0596$, Kruskal-Wallis statistics: 7.4229).

The β diversity, the difference between the diet groups, is presented through a principal coordinate analysis based on unweighted UniFrac distances (Fig. 3). The first and second principal coordinates are given. Axis 1 explains 43.7% of the variation, and axis 2 explains 22.6%. Distinct clusters separate the NCD and HFD + Ex groups. The HFD and HFD + Cal groups overlap, with the HFD + Cal group having a larger cluster than the HFD group.

Fig. 4A illustrates the relative abundance of the different bacterial phyla for each diet group. Firmicutes was the most abundant phylum in all 4 groups, followed by Bacteroidetes. There were no apparent differences in the relative abundance on the phylum level. But there were significant differences on the genus level as highlighted in Fig. 4B. It is obvious that the HFD and HFD + Cal groups share a similar bacterial profile, whereas the profiles of the HFD + Ex and NCD groups are different compared to HFD. To illustrate the relative abundance of the various genera in more detail, we also generated bar graphs ± standard error of the mean in Fig. 5. The most abundant genera in the feces samples were *Allobaculum*, *Lactobacillus*, *Lactococcus*, *Turicibacter*, and *Parabacteroides*. On first sight, it seemed that the main effect of HFD is a marked decline in *Allobaculum* and the presence of *Lactococcus* relative to the NCD group. Dietary supplementation with calanus oil did not seem to influence these HFD-induced changes in the microbiota composition, except for an apparently further decline in *Allobaculum* and an increased abundance of *Lactobacillus*. Treatment with exenatide reduced the high abundance in *Lactococcus* present in the HFD group. *Turicibacter* was abundant in all diet groups but mostly in the NCD and HFD + Ex group, whereas the presence of *Parabacteroides* seemed to be independent of diet.

A noncorrected 2-sided Student *t* test (comparing HFD vs NCD, HFD vs HFD + Cal, and HFD vs HFD + Ex) revealed that,

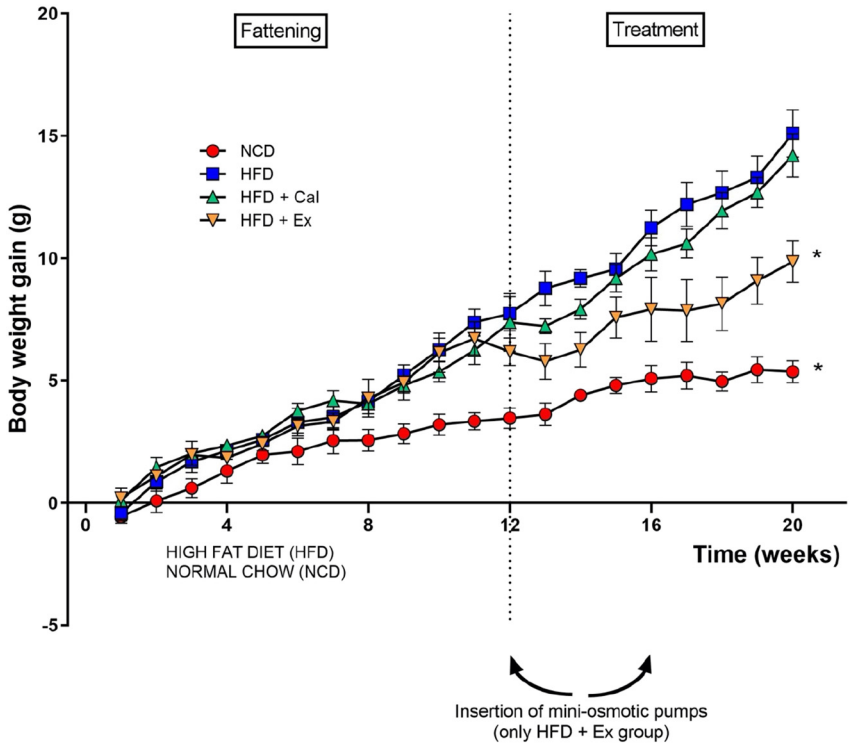

Fig. 1 – Body weight gain for the four different diet groups. Data are shown as means ± SEM. After 8 weeks of treatment, the weight gain of NCD and HFD + Ex groups was significantly different from HFD ($P < .05$).

indeed, the decrease in *Allobaculum* and increase in *Lactococcus* in the HFD compared to the NCD are significantly different, as was the increase in *Leuconostoc* (Fig. 6A). There were no significant differences between the HFD and HFD + Cal group. The apparent decrease in *Lactococcus* in the exenatide-treated group compared to the HFD group was significantly different, as well as the decrease in *Streptococcus* (Fig. 6B).

It is currently unknown at what abundance threshold certain gut bacteria lead to an increased disease risk. A statistical difference might therefore not necessarily lead to pathology or health improvement. Therefore, we visualize in Fig. 7 the differences of the various genera for each individual fecal sample in more detail with a heat map. Difference in colored tiles represents the multiplication of abundance of the genera, per sample, compared to the average abundance of those genera of all 20 fecal samples.

Table 4 – Content of PUFA in % of total fatty acid content in intra-abdominal fat from the various groups of mice

	SDA	DHA	EPA	AA
NCD	0.78 ± 0.15	0.26 ± 0.17	ND	0.42 ± 0.12
HFD	0.65 ± 0.03	0.19 ± 0.03	ND	0.40 ± 0.04
HFD + Cal	1.09 ± 0.12[a]	0.74 ± 0.10[a]	0.19 ± 0.04[a]	0.30 ± 0.04
HFD + Ex	0.71 ± 0.09	0.23 ± 0.04	ND	0.44 ± 0.09

Results are mean ± SD (n = 5 in each group).
[a] $P < .05$ vs HFD.

Some genera showed large variation in abundance within the diet groups (*Bacteroides*, *Parabacteroides*, *Lactobacillus*, *Akkermansia*, *Adlercreutzia*, *Coprococus*, *Blautia*, *Turicibacter*, *Bilophila*), whereas the abundance was more stable for other genera (*Lactococcus*, *Lauconostoc*, *Allobaculum*, *Ruminococcus*, *Streptococcus*, *Dorea*, *Oscillospira*). Thus, in line with the relative abundance shown in Fig. 5, the heat map shows that *Lactococcus*, *Leuconostoc*, and *Streptococcus* were above average present in the HFD and HFD + Cal groups, whereas *Dorea* and *Ruminococcus* were below average present in these groups. *Allobaculum* and *Oscillospira* were below average present in the 3 groups feeding on an HFD. Moreover, feces samples from the majority of mice in the HFD + Cal group were characterized by overrepresentation of *Lactobacillus* and *Streptococcus* and underrepresentation of *Bilophila*. In the HFD + Ex group, overrepresentation of *Ruminococcus* was particularly evident.

3.3. *Metagenomic functional prediction*

The 16S rRNA gene was used as a marker gene to extrapolate the taxonomic findings into functional predictions. The taxonomic information from screening samples against the Greengenes reference database was used to extract information from the complete genome sequences of the identified species. Combining whole genome information together with abundance measures for each species allowed for the prediction and quantification of functional pathways. Statistical analysis of the Kyoto Encyclopedia of Genes and Genomes data identified in total 328 genes related to

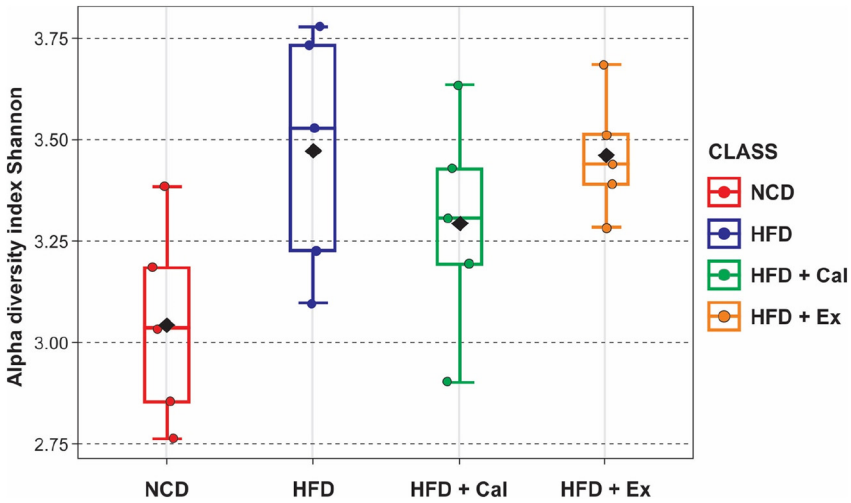

Fig. 2 – *α* diversity measured as Shannon Phylogenetic Index on OUT level for the 4 different diet groups. There were no statistically significant changes in the *α* diversity between the groups. (P = .0596, Kruskal-Wallis statistics: 7.4229).

functional categories (proteins) (Supplementary Table S1). Significant differences in the relative abundance of the predicted microbial genes (based on the noncorrected 2-sided Student t test) revealed the difference in the mean proportions of these genes between the HFD and NCD groups, the HFD and HFD + Cal groups, and the HFD and the HFD + Ex groups (Figs. 8A-C). As can be seen in Fig. 8A, 38 categories were differently expressed in the HFD vs NCD groups. Relevant for the current study was the overexpression of lipid and fatty acid biosynthesis proteins in the gut microbiota of the HFD-fed mice. Fig. 8B gives the 7 functional categories that were differently present in the colonic microbiota in the HFD and HFD + Cal groups. The novel marine oil upregulated 4 of the 7 categories in the high-fat–fed mice, which included proteins related to glycolysis, gluconeogenesis, and phosphate transferase systems (Fig. 8B). Ten categories where differently expressed in the microbiota of the HFD vs the HFD + Ex group (Fig. 8C). With respect to metabolic control, we note that proteins involved in glycine, serine, and threonine metabolism were overexpressed in the HFD + Ex group.

3.4. *Gene expression*

The gene expression of several markers for inflammation, fat storage regulation, and permeability of the intestinal wall

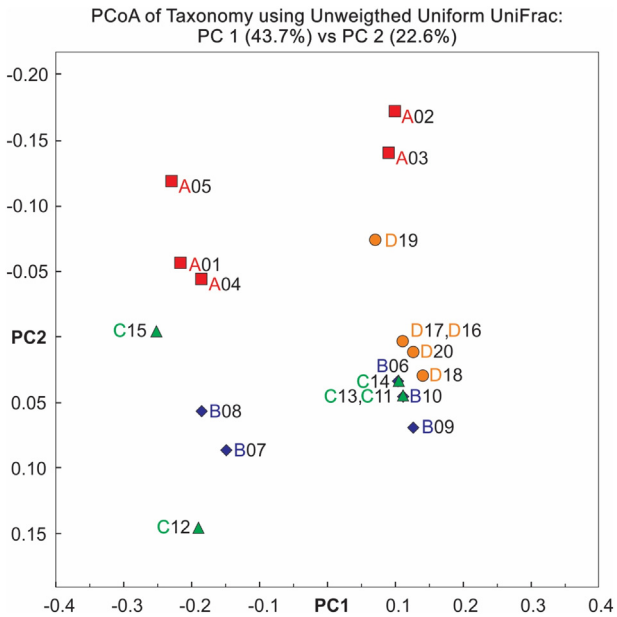

Fig. 3 – Principle coordinate analysis based on unweighted UniFrac in mice fed different diets. The symbols indicate values for individual mice. Percent variation explained by the principal coordinates PC 1 and PC 2 was 43.7% and 22.6%, respectively. Red squares, NCD; blue diamonds, HFD; green triangles, HFD + Cal; orange circles, HFD + Ex.

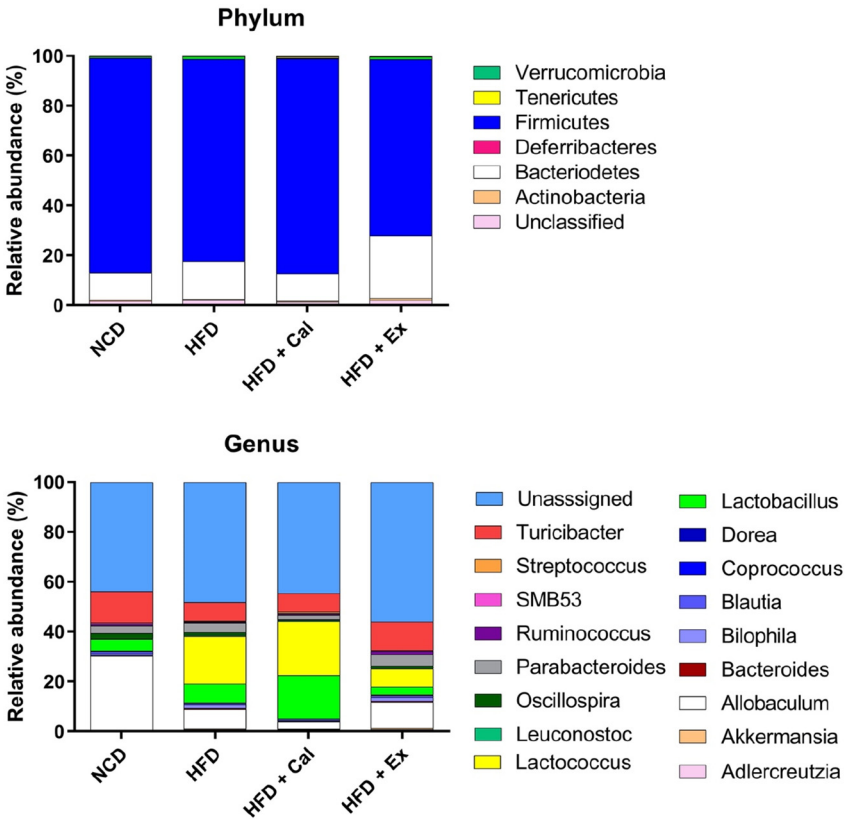

Fig. 4 – Relative abundance at the phyla (A) and genera levels (B) in the 4 diet groups.

were measured in the omental WAT (GPR120, IL-1β, IL-6, IL-10, TNF-α, CD36, EMR1a, adiponectin TLR4, MCP1, NF-κB, FIAF, PPARα, PPARβ, PPARγ, and GLP2 R) and the colon (GPR41, GPR43, GPR120, TNF-α, MUC2, GLP2 R, IL-1β, Il-10, IL-18, IFN-γ, FIAF, ZO-1, occluding, TLR4, and NF-κB). None of the analyzed genes were up- or downregulated depending on diet in the 2 tissues, with the exception of adiponectin (see also Supplementary Figs. S1 and S2).

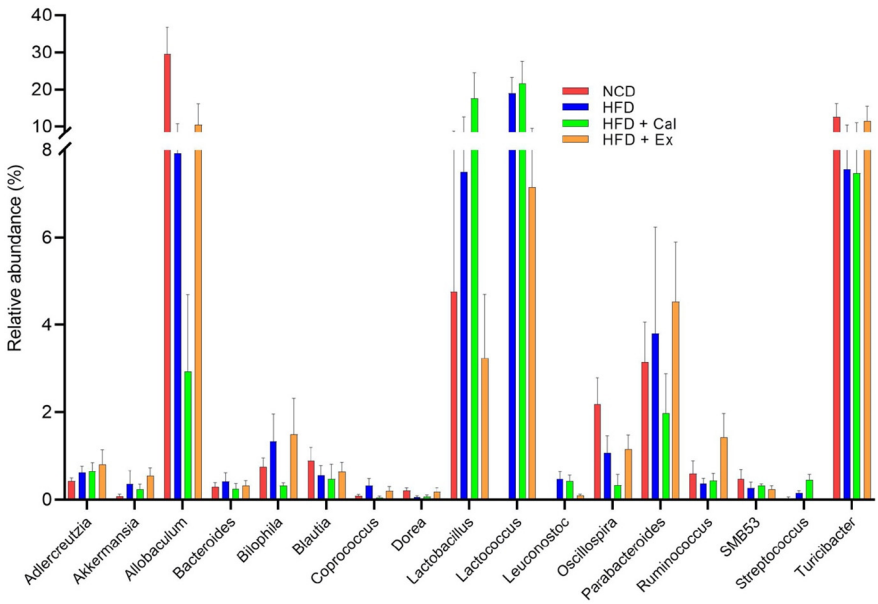

Fig. 5 – Relative abundance of the different genera present in colon feces samples from the 4 diet groups. Data shown as means ± SEM.

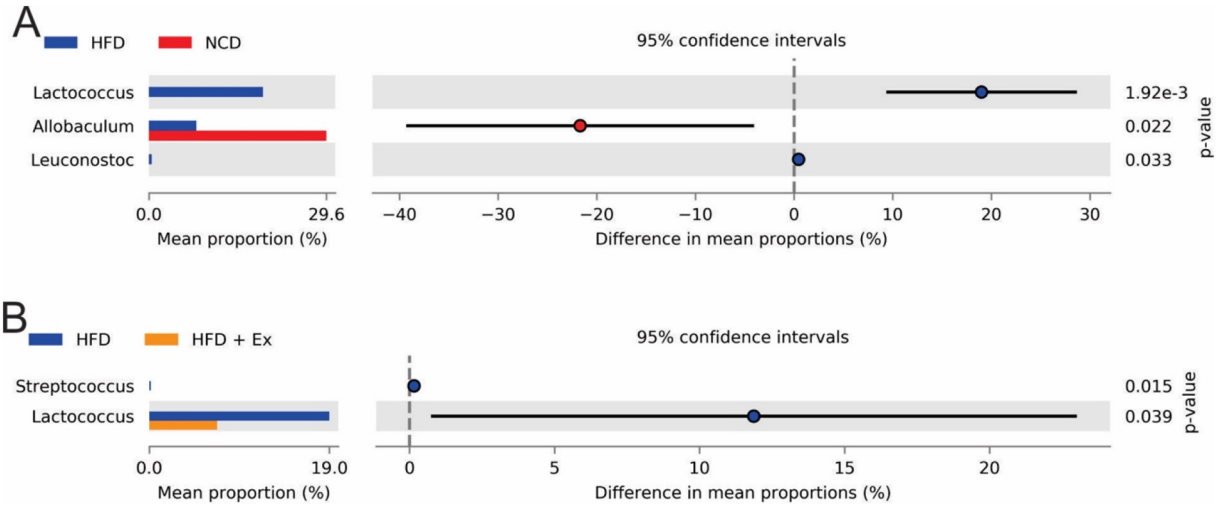

Fig. 6 – Comparison of mean proportion of bacterial genera. (A) HFD vs NCD groups. (B) HFD vs HFD + Ex groups. Only statistically significant differences ($P < .05$, noncorrected 2-sided Student t test) are shown.

4. Discussion

Diet has long been considered the most important driver behind changes in the intestinal microbiota [7-9]. In this study, we show that long-term feeding on a HFD led to an increase in the intestinal abundance of the bacterial genera *Lactococcus* and *Leuconostoc*, whereas the abundance of *Allobaculum* and *Oscillospira* was decreased. Supplementation of the HFD with 2% of wax ester–containing oil from *C*

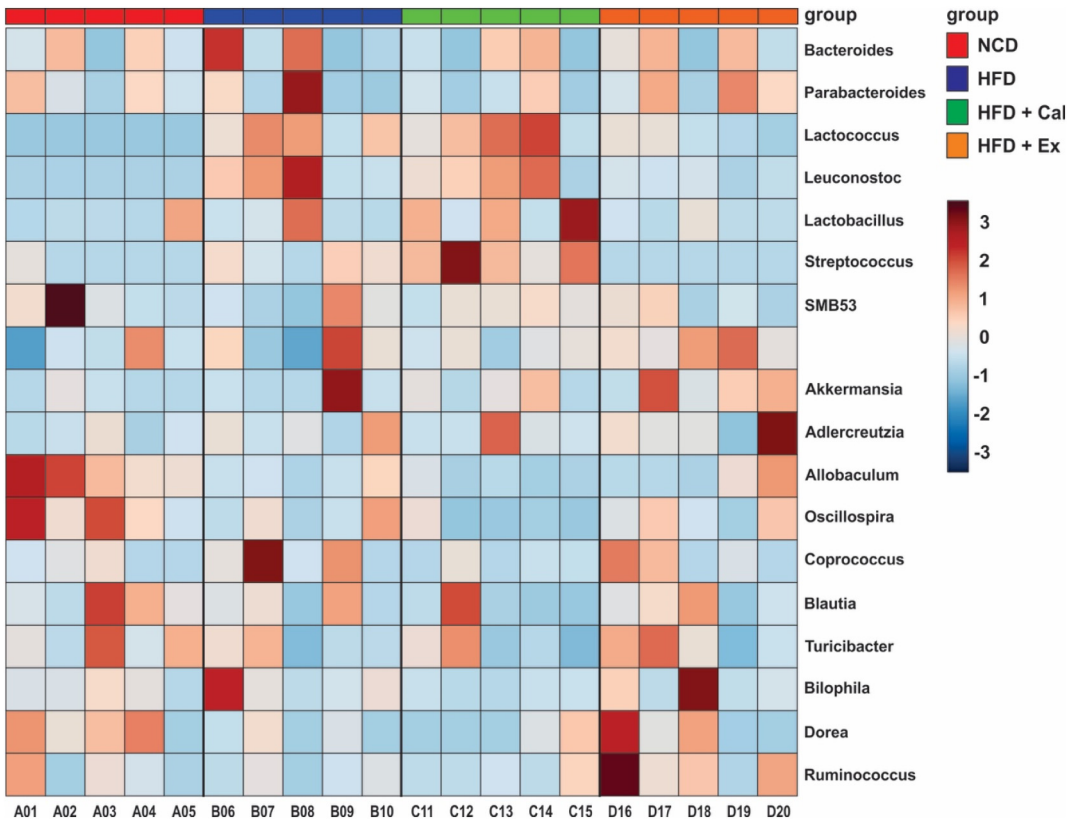

Fig. 7 – Heat map showing the representation of the different genera in each individual fecal sample compared to the average abundance of those genera. The multicolored bar at the top delineates the 4 diet groups, whereas the individual samples are indicated at the bottom of the heat map. The colored tiles indicate a positive (light to dark brown) or negative multiplication (light to dark blue) of the genus average across all individual samples.

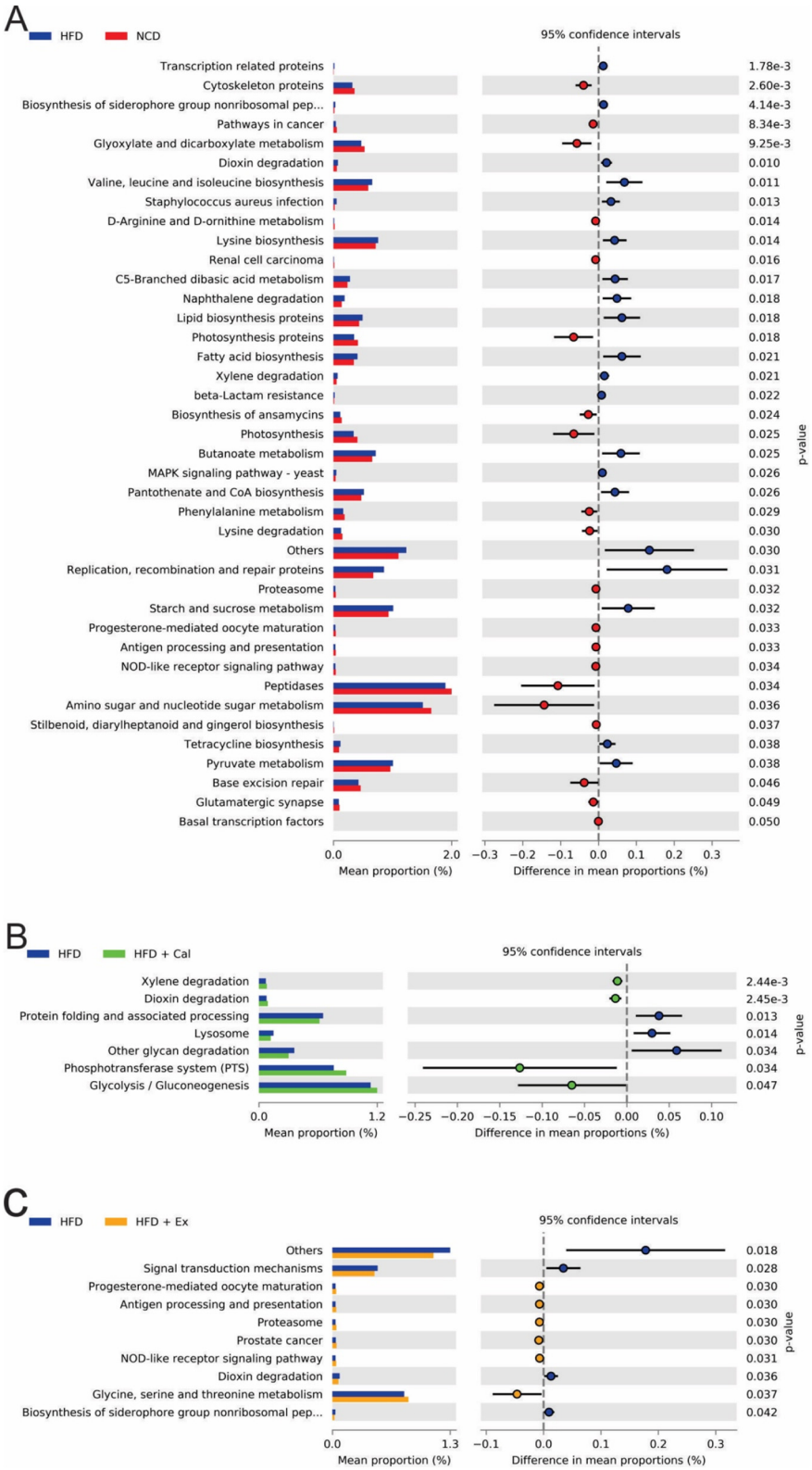

Fig. 8 – Comparison of mean proportion of predicted microbial genes present in colonic samples related to functional categories of (A) HFD vs NCD, (B) HFD vs HFD + Cal, and (C) HFD vs HFD + Ex. Only significant ($P < .05$) functional pathways are shown based on a noncorrected 2-sided Student t test. The colored circles represent the difference in mean proportions of the various functional categories with the 95% confidence interval, as calculated using bootstrap method.

finmarchicus did not alter this pattern significantly, although it led to an overrepresentation of *Lactobacillus* and *Streptococcus* and underrepresentation of *Bilophila*. Administration of the GLP-1 analog exenatide prevented the HFD-induced increase in *Lactococcus*, and in addition, it caused a decrease in the abundance of *Streptococcus*.

The high relative abundance of *Lactococcus* and the near depletion of *Allobaculum* in the HFD group are in agreement with previous animal studies [8,9]. *Lactococcus* is associated with proinflammatory markers [8,13,15], whereas *Allobaculum* is a short-chain fatty acid–producing bacterium which is considered health-promoting. Therefore, HFD used in the current study seems to change the microbiota composition in an unhealthy direction. This view was further supported by the finding that *Oscillospira*, a genus of importance for human health [37] and which has been reported to correlate negatively with body mass index and paracellular permeability in humans [38], was depleted in nearly all HFD mice.

Dietary fat has been shown to modulate gut microbiota [39], and to our knowledge, this is the first study investigating the effect of the oil from *C finmarchicus* on gut microbiota. As mentioned in the introduction, this marine oil contains more than 85% wax esters, consisting of saturated, monounsaturated, and polyunsaturated fatty acids bound to long-chain fatty alcohols. The general view is that the omega-3 fatty acids EPA and DHA represent the active substance in marine oil preparations, but the high content of SDA (7%) and fatty alcohols (about 350 mg/g), which is unique for this novel marine oil, could also be beneficial for human health, [40]. To which extent this fatty acid and fatty alcohols affect the composition of the gut microbiota is, to our knowledge, not known.

Supplementing HFD with 2% calanus oil was not able to restore the microbiota composition to that of the NCD group. Nevertheless, the relative abundance of *Lactobacillus* and *Streptococcus* was above average in the majority of the mice receiving the marine oil. This observation is in line with results by Caesar et al. [14], who reported increased abundance of *Lactobacillus* and *Streptococcus* when they replaced the lard in the high-fat diet with fish oil (Menhaden oil). Our results are also in line with findings by Mujico et al [41], who observed an increase in *Lactobacillus* following supplementation of HFD with EPA and DHA in female mice. Caesar et al. [14] reported reduced body weight gain and reduced inflammation due to an interaction between dietary lipids and the microbiota, which reduced toll-like receptor 4 (TLR4)–induced secretion of monocyte chemoattractant protein-1 (MCP-1/CCL2). Unlike the study by Caesar et al. (2015), we did not detect any differences between the groups with respect to WAT or colon gene expression of MCP-1, TLR4, and other inflammatory markers, which could be due to a relatively short treatment period with the marine calanus oil used in the present study. We observed an antiobesogenic effect of the wax ester–rich oil in terms of a reduced adiposity index. The mice did not, however, show a clear reduction in body weight development, as seen in previous experiments [26,27], and this could be due to a relatively short treatment period (8 vs 27 weeks). In addition, it should be noted that we used female mice in the current experiment and therefore cannot exclude gender differences with respect to the response of the marine oil supplementation. We did however observe an increased level of n-3 PUFA (and decline in n-6 PUFA) in intra-abdominal fat of mice receiving HFD supplemented with calanus oil, which is in line with previous results [18] and demonstrates very clearly that the wax ester in the oil is digested in the intestine is taken up, and thereby contributes to a healthier adipose tissue. In light of the role of omega-3 fatty acids as PPAR ligands [42] and the consequent activation of gene transcription, it was a bit surprising that the reduced adiposity index following calanus oil supplementation was not reflected in any significant change in PPAR expression in adipose tissue. One possibility could be that PPARα was upregulated primarily in metabolically active tissues, such as liver, heart, and skeletal muscle, leading to increased fatty acid oxidation and thereby draining of fatty acids from WAT.

The increased abundance of *Lactobacillus* might be of biological relevance because it is considered a health-promoting, short-chain fatty acid–producing probiotic strain that is often associated with weight loss, regulation of fat metabolism, and anti-inflammation (as reviewed in [13] and [43]). Most species of *Streptococcus* are considered pathogenic, but, for example, *Streptococcus thermophilus* is a probiotic. Unfortunately, our analysis was not capable of distinguishing between the different species of this genus, so the impact of the increased abundance of *Streptococcus* is not clear. Caesar et al. [14] also found a lower abundance of *Bilophila* in the fish oil–fed mice compared to the lard-fed mice. Interestingly, all 5 animals fed HFD + Cal in the current study also showed a below average abundance of *Bilophila*. This genus is shown to increase upon consumption of diets rich in saturated animal fats, and increased abundance of *Bilophila wadsworthia* is associated with inflammatory bowel disease [44]. To find out if the decreased adiposity index in response to dietary oil, as observed in the present study, was due to an enrichment of *Lactobacillus* and/or depletion of *Bilophila*, a larger study needs to be done.

In recent years, the effect of antidiabetic drugs on the gut microbiota has received more and more attention both in humans and in animal models. For example, it is known that the most used antidiabetic drug, metformin, as well as different α-glucosidase inhibitors (acarbose, miglitol, and voglibose), alters the composition of the gut microbiota [45,46]. A few studies examined the effect of liraglutide which, like exenatide, is a GLP-1 receptor agonist [47-49]. Treatment with liraglutide was associated with an increase in *Blautia* and *Turicibacter*, whereas the effect on *Allobaculum* and *Lactobacillus* was less clear and dependent on the animal model in question [47,48]. Wang et al. [47] found that liraglutide-induced weight loss was associated with, among others, *Lactobacillus*, *Turicibacter*, *Coprococcus*, and *Blautia*. The exenatide-treated mice in our study showed decreased body weight and fat mass compared to the HFD mice, and in line with Wang et al. [47], we observed a slight increase in the abundance of *Turicibacter* in this group. However, we did not observe any effect of exenatide on the other "weight reducing" bacteria. Interestingly, exenatide seemed to reduce the abundance of *Streptococcus* and *Lactococcus*, which are considered pathogenic, while increasing the abundance of *Ruminococcus* and *Dorea*. The latter 2 genera are generally related to insulin resistance and disturbed metabolic health [50,51]. In addition, De Filippis et al. [52] found a correlation between *Ruminococcus* and trimethylamine N-oxide levels, a microbiota-dependent metabolite derived

from trimethylamine-containing nutrients that are abundant in a Western diet and has been associated with obesity, insulin resistance, and increased thrombosis potential in animal and clinical studies [53]. Thus, the previously reported improvement in insulin sensitivity following exenatide treatment [28] could imply the effect of reduced abundance of *Streptococcus* and *Lactococcus* overrides that of the increase in *Ruminococcus* and *Dorea*.

It is currently unknown how GLP-1 receptor agonists induce alterations in the microbiota profile, but changes in pH and nutrient composition in the gut, as well as delay in gut transit time and gastric emptying rate, could be involved [47]. Another proposed mechanism is via the interplay with bile acids [51]. GLP-1 receptor agonists are suggested to induce weight loss via reduced food intake due to inhibition of appetite and reduced gastric emptying. In our study however, we could not detect any difference in food intake between the HFD-fed groups. We therefore suggest that weight loss in response to exenatide treatment could be due to a re-establishment of the abundance of *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Turicibacter*, *Dorea*, and *Ruminococcus* which characterized the lean NCD group.

Functional analysis of the gut microbiota revealed significant changes between the NCD- and the HFD-fed mice. Mice fed an HFD had increased abundance of bacteria with capacity for lipid and fatty acid biosynthesis, that is, substrates which could become available as nutrients or bioactive compounds for the host and explain the observed increase in body weight and adiposity index in this group. The microbial composition of the HFD + Cal group was associated with increased activities of the phosphotransferase system, glycolysis, and gluconeogenesis compared to the HFD mice. These 2 functions enable the bacteria to take up and metabolize carbohydrates, producing metabolites which could potentially be used by the host. To determine if this is related to the reduced adiposity index in the mice fed oil from *C finmarchicus*, a further in-depth study is needed. Functional analysis of the microbiota from HFD + Ex mice showed upregulation of signal transduction pathways, but the implication of this observation also needs further studies.

This is the first study examining the effect of calanus oil, a wax ester–containing oil derived from *C finmarchicus*, on the gut microbiota composition. Obesity in female mice was associated with an enrichment of the proinflammatory *Lactococcus* and a depletion of the anti-inflammatory and health-promoting *Allobaculum* and *Oscillospira*, changing the microbiota composition in an unhealthy direction. Although dietary oil from *C finmarchicus* was not able to restore the microbiota composition to that of the lean control group, it resulted in a relatively high abundance of *Lactobacillus*, a health-promoting genus, which is often related to weight loss. It also reduced the abundance of the pathogenic *Bilophila* genus. Treatment with exenatide partly restored the bacterial profile found in the lean control group and seemed to reduce the abundance of *Streptococcus* and *Lactococcus*, which are both considered pathogenic. These data confirm our hypothesis, namely, that dietary supplementation with the antiobesogenic calanus oil is able to antagonize the unfavorable changes in the gut microbiota induced by high-fat feeding.

Differences in sample preservation and DNA extraction, library preparation, as well as use of different reference databases or software programs make it difficult to compare results from one laboratory to another [54-56]. In this study, the number of animals in each group was relatively small (n = 5), and hence, the power of the statistical analysis was relatively low. In addition, determination of the microbiota composition was based only on feces samples from the colon.

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Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: TSL has a small position as scientific advisor in Calanus AS. AMP is employed as product manager in Calanus AS. PCS, KMJ, RLO, and JM declare no conflict of interest.

Supplemental materials

Supplemental materials were provided that include diet information; Table S1, Overview of all identified functional categories; Figure S1, Gene expression of markers related to inflammation, intestine permeability, and fat metabolism in the colon of female mice; and Figure S2, Gene expression of markers related to inflammation and fat metabolism in omental WAT of female mice. Supplementary data to this article can be found online at https://doi.org/10.1016/j.nutres.2020.09.002.

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Review:

Obesity-induced alterations in the gut microbiome in female mice fed a high-fat diet are antagonized by dietary supplementation with a novel, wax ester– rich, marine oil

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Review

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Impact of Obesity-Related Inflammation on Cardiac Metabolism and Function

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ABSTRACT

This review focuses on the role of adipose tissue in obese individuals in the development of metabolic diseases, and their consequences for metabolic and functional derangements in the heart. The general idea is that the expansion of adipocytes during the development of obesity gives rise to unhealthy adipose tissue, characterized by low-grade inflammation and the release of proinflammatory adipokines and fatty acids (FAs). This condition, in turn, causes systemic inflammation and elevated FA concentrations in the circulation, which links obesity to several pathologies, including impaired insulin signaling in cardiac muscle and a subsequent shift in myocardial substrate oxidation in favor of FAs and reduced cardiac efficiency. This review also argues that efforts to prevent obesity-related cardiometabolic disease should focus on anti-obesogenic strategies to restore normal adipose tissue metabolism.

Keywords: Visceral adipose tissue; Inflammation; Lipid metabolism; Heart; Oxygen consumption

INTRODUCTION

Obesity causes adverse metabolic effects and is a major risk factor for metabolic diseases, such as type 2 diabetes and fatty liver disease, which increase the risk of coronary heart disease (CHD) and ischemic stroke. Obesity is a growing health problem in both developed and developing countries, and in the last 20 years the world has witnessed an alarming increase in obesity.[1] Obesity has nearly tripled worldwide since 1975, and according to the World Health Organization, more than 1.9 billion adults (18 years and older) were overweight in 2016.[2] Of these, over 650 million were obese (defined as a body mass index above 30 kg/m^2). It should also be noted that 38 million children under the age of 5 were overweight or obese in 2019. In China, the world's most populous country, obesity has also increased at an alarmingly rapid rate, and during the past decade the prevalence of obesity in the country has tripled, while that of abdominal obesity has increased by more than 50%.[3] These numbers are expected to rise in the future unless effective actions are taken to prevent such a development.[2] The current rise in human obesity is primarily linked to increased energy intake and decreased energy expenditure, resulting in excess fat deposition in adipose tissue.[4]

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ADIPOSE TISSUE: AN ENERGY RESERVOIR WITH THE CAPACITY TO CHANGE ITS DIMENSIONS IN RESPONSE TO NUTRITIONAL STATUS

Storage of extra energy obtained during food abundance in adipose tissue is an essential physiological activity in living organisms, especially in free-ranging animals who have to deal with marked seasonal alterations in food availability.[5] Fat storage is also important in humans in order to maintain metabolic homeostasis during the post-prandial period, and even more importantly, in humans undergoing extended periods of starvation. The adipose tissue is distributed throughout the body and has a large capacity to expand to accommodate excess energy in the form of lipids. White adipose tissue comprises two major depots, subcutaneous and visceral adipose tissue, the latter of which is found within the abdominal cavity and stored around important internal organs. Anatomically, it is further subdivided into mesenteric, omental, perirenal, and peritoneal depots.[6,7] Although adipose tissue historically has been regarded as an energy storage depot, research over the last few decades has revealed that adipose tissue also acts as an endocrine organ. Thus, several cytokines, hormones, and peptides secreted by adipocytes, collectively termed as "adipokines" (e.g. leptin, resistin, adiponectin, tumor necrosis factor alpha [TNF-α], and interleukin [IL]-6) have been identified and intensively investigated to elucidate their roles in the control of energy homeostasis.[8,9]

Subcutaneous adipose tissue is the largest fat depot in the body. The expansion of subcutaneous adipose tissue occurs through the recruitment and differentiation of adipose precursor cells, resulting in healthy adipose tissue.[10] However, when the storage capacity of the subcutaneous depot is exceeded, excess energy intake leads to fat accumulation in undesirable locations, such as the intra-abdominal depots, as well as in ectopic tissues such as the liver, skeletal muscle, and heart. Over time, this situation creates a condition commonly referred to as "lipotoxicity," as described in more detail below.

LOW-GRADE INFLAMMATION IN OBESE ADIPOSE TISSUE

Adipose tissue is considered to be a pathogenic site of obesity-induced insulin resistance.[11] This is due to the fact that adipose tissue in obese individuals, particularly those with abdominal obesity, is associated with a chronic, local low-grade inflammatory response involving the production of pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) and chemokines.[4,12-14] Numerous studies have shown that cellular stress is a major factor contributing to inflammation in adipose tissue.[4,15] Thus, in response to nutrient excess, adipocytes expand and become hypertrophic. At the same time, the distance between blood-bearing vessels increases and oxygen diffusion becomes insufficient,[16] leading to local hypoxia, which triggers the increased secretion of inflammatory markers.[17] Characteristically, the adipose tissue of obese individuals shows lower blood flow, higher vasoconstriction, and lower capillary density than adipose tissue in non-obese individuals.[15]

Macrophage infiltration is another characteristic of adipose tissue in obese individuals. After initial rolling and attachment of monocytes to activated endothelial cells, monocytes then extravasate through the endothelial cell layer and differentiate into macrophages. It has been reported that monocyte chemoattractant protein-1 (MCP-1) secretion is markedly enhanced locally and in the plasma of obese rodents and humans.[18,19] At the onset of an inflammatory

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process, the macrophages that are usually present in the adipose tissue switch from an anti-inflammatory (M2) state to a pro-inflammatory (M1) state.[20] More than 90% of M1-type macrophages are localized to dead adipocytes and form so-called "crown-like structures," which are a characteristic component of the immuno-histological picture of adipose tissue in both obese mice and humans.[16] Cross-talk between adipocytes, macrophages, and endothelial cells may enhance the inflammatory state by increasing the secretion of pro-inflammatory cytokines and chemokines, which in turn can develop into local and/or systemic insulin resistance in a paracrine and/or endocrine fashion.

Sun et al.[17] documented increased interstitial fibrosis in white adipose tissue during the development of obesity, which may reduce extracellular matrix flexibility and decrease the tissue plasticity, ultimately leading to adipocyte dysfunction. Abnormal collagen deposition, which characterizes fibrosis development in adipose tissue, is paralleled by the infiltration of macrophages and other immune cells.[21] Under these conditions, fibrotic response genes are markedly up-regulated, and classically activated pro-inflammatory M1 macrophages are attracted by dead adipocytes,[17] reinforcing the inflammatory process and altering adipose tissue metabolism. Thus, the development of hypertrophic adipose tissue (in response to excess energy intake), macrophage infiltration, and fibrosis are major factors initiating the local low-grade inflammatory response in adipose tissue. On the molecular level, this process includes activation of the c-Jun N-terminal kinase (JNK) and IκB kinase (IKK) β/nuclear factor kappa light chain enhancer of activated B cells inflammatory signaling pathways,[22] which in turn regulate protein phosphorylation and cellular transcriptional events leading to the secretion of proinflammatory cytokines (TNF-α, IL-6, and IL-1β) and chemokines, such as MCP-1.[23]

INFLAMMATION AND LIPID OVERLOAD CAUSE DYSREGULATION OF MYOCARDIAL METABOLISM AND VENTRICULAR FUNCTION

Low-grade inflammation in abdominal adipose tissue also contributes to hepatic inflammation due to portal delivery of abdominal fat—derived cytokines and lipids.[11,16] Thus, TNF-α and IL-6 originating from adipocytes, as well as from macrophages, in adipose tissue and the liver[22] create systemic inflammation and subsequent dysregulation of insulin action in peripheral tissues, such as skeletal and cardiac muscle[24] (**Fig. 1**).

Although the role of inflammation in the etiology of myocardial insulin resistance is limited, Ko et al.[25] reported that high-fat feeding of rats caused increased macrophage infiltration in myocardial tissue from these animals, as well as increased cytokine and suppressor of cytokine signaling proteins levels in cardiomyocytes. These observations were associated with reduced myocardial insulin sensitivity and glucose metabolism. It was proposed that cytokines from macrophages and cardiomyocytes activate their receptors and associated signaling pathways to increase serine phosphorylation of insulin receptor substrate 1 (IRS-1). This eventually leads to insulin resistance via inhibition of protein kinase B/Akt and reduced glucose transporter type 4 (GLUT4) translocation.[26]

Increased uptake of fatty acids (FAs) also plays a central role in the development of cardiac insulin resistance in obesity. Increased FA uptake is catalyzed, in part, by the translocation of FA transporters (FAT/CD36) to the sarcolemma.[27-29] However, not all FAs entering the cell are

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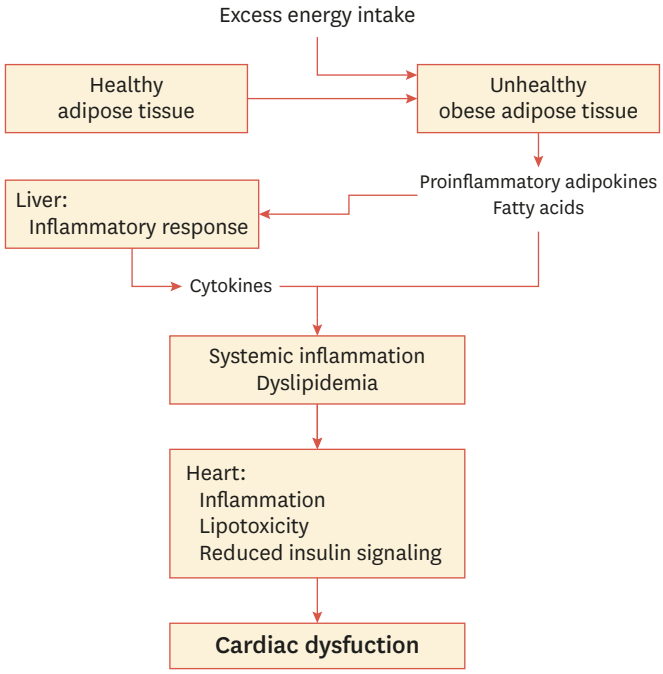

Fig. 1. Increasing visceral obesity causes inflammatory responses and metabolic dysregulation in fat and liver tissue. This condition involves infiltration of monocytes and macrophages and subsequent secretion of pro-inflammatory adipokines and elevated release of free fatty acids, leading to systemic inflammation, which promotes insulin resistance in several organs, including the heart. In addition, an elevated supply of lipids (free and esterified fatty acids) exceeds the fatty acid oxidation capacity and causes lipotoxicity in the myocardium, eventually leading to cardiac dysfunction.

utilized for oxidative purposes, and long-chain FAs in the form of acyl-CoA provide substrates for nonoxidative processes such as triglyceride, diacylglycerol, and ceramide synthesis.[30,31] The accumulation of these substances is known to activate kinases, including JNK, IKK, and protein kinase C, which down-regulate insulin signaling[32,33] via serine phosphorylation of IRS-1.[27,34] Besides its adverse effects on insulin signaling and glucose metabolism, excessive lipid accumulation may also have direct lipotoxic effects on cardiomyocytes.[30,35]

The mismatch between FA uptake and oxidation by cardiomyocytes[27,28] and the consequent myocardial lipid accumulation and insulin resistance may have serious cardiac consequences that ultimately lead to compromised cardiac mechanical function.[35,36] Thus, reduced left ventricular (LV) systolic function has also been demonstrated in several animal models of obesity,[37-39] except for some studies in diet-induced obese rats that showed unchanged or mildly reduced systolic function.[40,41] FA binding protein 4, an intracellular lipid-binding protein involved in the transportation of FAs, has been suggested to be strongly associated with inflammation, obesity, diabetes, and cardiovascular diseases (CVD).[42] Cardiac-specific overexpression of this protein in mice resulted in greater cardiac hypertrophy following transverse aorta constriction than in wild-type controls.[43] Furthermore, transgenic mice expressing mutated lipoprotein lipase (GPI-anchored human LPL) in cardiomyocytes developed dilated cardiomyopathy with lipid accumulation within myocytes.[44] Mice with cardiomyocyte-restricted knockout of the insulin receptor also exhibited reduced heart size and mildly impaired contractile function, indicating that insulin signaling is an important physiological regulator of growth and function.[45,46]

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Many studies have demonstrated that obesity (isolated or co-existing with hypertension) in humans is associated with abnormal diastolic function,[47-49] whereas impairment of systolic function is not consistently observed.[50] Obesity-related dysfunction includes left heart remodeling (i.e., left atrial dilatation and LV hypertrophy) as well as abnormalities in LV contractile and relaxation functions (i.e., LV stiffness and impaired relaxation).[47,51-53] This condition can ultimately progress to cardiac hypertrophy and/or systolic dysfunction when lipotoxicity and/or local perfusion heterogeneities result in cell death and fibrosis.[36,54-56]

OBESITY-INDUCED ALTERATIONS IN MYOCARDIAL SUBSTRATE UTILIZATION: LOSS OF METABOLIC FLEXIBILITY

Approximately 50%–70% of the energy (ATP) requirement of the healthy heart is produced by oxidation of long-chain FAs, which are bound to albumin or esterified in circulating triglycerides, whereas carbohydrates, lactate, and to some extent also ketone bodies and amino acids account for the rest of overall ATP production.[57,58] Although the normal heart seems to prefer FAs for the production of energy, it has the ability to change to other substrates for the generation of ATP to ensure that its energy demands are met. The contribution of individual substrates to ATP production depends on substrate availability, hormonal status, and energy demand, and the capacity of the heart to switch between the different energy substrates is referred to as "metabolic flexibility." In the 1960s, Sir Philip Randle performed landmark studies showing how metabolic products of increased FA oxidation can inhibit glucose uptake in muscle.[59] This mechanism, subsequently known as the Randle cycle, is the basis of metabolic flexibility in healthy individuals, which allows energy-requiring organs such as heart and skeletal muscle to switch between fuels, depending on nutrient composition and intake, as well as variations in insulin signaling. As mentioned above, the substrate transporters GLUT4 (for glucose) and CD36 (for FAs), play a central role in this dynamic balance of substrate utilization.[60] CD36 plays a central role in facilitating cellular long-chain FA uptake across the plasma membrane, acting in concert with other membrane proteins, such as FA-binding protein.[61] With the development of insulin resistance, however, the metabolic flexibility of the heart (as well as skeletal muscle) deteriorates,[55] so that myocardial energy production becomes primarily dependent on FA oxidation. As a consequence, accumulation of the intermediates of FA metabolism in cardiomyocytes results in a state of lipotoxicity (as discussed above),[30,56] causing cellular oxidative stress, impaired cytosolic and mitochondrial calcium homeostasis, and mitochondrial dysfunction.

ACUTE AND SUSTAINED ELEVATIONS OF THE FA SUPPLY LEAD TO INCREASED MYOCARDIAL OXYGEN CONSUMPTION (MVO_2) AND IMPAIRED ENERGETICS

A study conducted in the beginning of the 1970s[62] using a canine model reported that MVO_2 increased markedly in response to acute elevations in the plasma concentration of FA. In addition, higher FA oxidation and MVO_2 were reported in obese relative to non-obese young women.[63] It has been suggested that uncoupling of oxidative phosphorylation and induction of energy-wasting triglyceride-FA[64,65] and Ca^{2+} cycling[66] could contribute to this elevation in MVO_2. Moreover, it was proposed that an excess substrate supply might result in impaired transcriptional regulation of proteins involved in the pathways of cardiac energy

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metabolism.[67] Thus, it was reported that patients undergoing coronary artery bypass graft surgery exhibited elevated plasma FA concentrations, which were associated with higher expression of cardiac mitochondrial uncoupling proteins.[67] Moreover, an impaired cardiac energy reserve in patients with type 2 diabetes mellitus (as reflected by a lower myocardial phosphocreatine [PCr]/ATP ratio) was correlated with fasting plasma FA concentration,[68] a finding that is also in line with increased mitochondrial uncoupling. Cardiac PCr/ATP ratios have also been documented during catecholamine stress[69] or exercise[70] in people with obesity and insulin resistance, although this response is not always observed.[55] Whether a lower myocardial PCr/ATP ratio in diabetic cardiomyopathy is a cause or effect of the progression to heart failure is currently unknown.[71]

Cardiac efficiency is characterized by the relationship between the mechanical performance and energy consumption of the heart in the form of ATP utilization or oxygen consumption. The development of the pressure-volume conductance catheter enabled calculation of the total work performed by the heart during the cardiac cycle as the pressure-volume area (PVA), and the relationship between MVO_2 and PVA can be used to calculate the oxygen used for mechanical activity versus the oxygen consumed for basal metabolism and excitation-contraction coupling. Oxygen consumption for the latter 2 processes is achieved by extrapolating the MVO_2-PVA relationship to zero work and is referred to as unloaded MVO_2.[72] Around the turn of the 21st century, Korvald et al.[73] showed, for the first time, that the MVO_2-PVA relationship was significantly influenced by changes in myocardial substrate metabolism in pigs. Thus, a change in myocardial metabolism from glucose towards higher FA oxidation shifted the *in vivo* MVO_2-PVA relationship upward in a parallel manner, reflecting that hearts exposed to high levels of FAs used more energy, independent of the workload. This elevation in MVO_2 was ascribed to a higher unloaded MVO_2 (i.e., the use of more oxygen for basal metabolism and excitation-contraction coupling), and the increased ratio between MVO_2 and work was translated into decreased cardiac efficiency. Similar observations were reported by How et al.[74] using isolated perfused working mouse hearts exposed to different workloads. In the same manner as in pigs, elevation of the FA concentration in the perfusion buffer shifted the MVO_2-PVA relationship upward, producing a near 30% increase in unloaded MVO_2. It should be noted that the FA-induced elevation in MVO_2 can by no means be explained by the switch in metabolism from glucose to FAs, since the difference in the phosphorylation-to-oxidation (P/O) ratios between FA and glucose oxidation (2.33 vs. 2.58, respectively) could account for a maximum increase in oxygen consumption of 11%. Other mechanisms, such as uncoupling of oxidative phosphorylation in the mitochondria and induction of futile cycles, as discussed above, could explain the high MVO_2 during predominant FA utilization. In line with this notion, Cole et al.[75] reported a lower mitochondrial maximal respiratory capacity and efficiency (P/O ratio) in high-fat–fed rats and suggested that decreased respiratory coupling can contribute to the impaired cardiac efficiency observed following obesity.

CHANGES IN CARDIAC METABOLISM AND FUNCTION IN OBESE AND DIABETIC ANIMALS RESULT IN REDUCED CARDIAC EFFICIENCY

Over the years, our laboratory has studied energy metabolism and cardiac performance of *ex vivo* perfused hearts from type 2 diabetic (*db*/*db*) as well as diet-induced obese mice. In accordance with other researchers,[76,77] we have demonstrated repeatedly that hearts from

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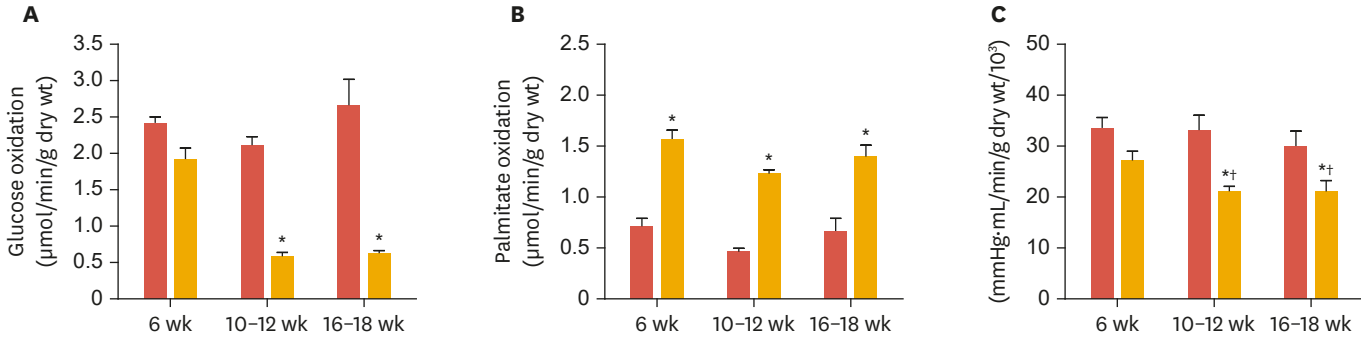

Fig. 2. Age-dependent changes in myocardial substrate oxidation and ventricular function in control (*db*/+, red columns) and type 2 diabetic (*db/db*, yellow columns) mice. (A) Reduction of glucose oxidation in *db/db* hearts after 10–12 weeks, while fatty acid oxidation had already significantly increased at 6 weeks (B), preceding the decline of left ventricular function (C), measured as PSP times CO. Modified from Aasum et al.[51]
PSP, peak systolic pressure; CO, cardiac output.
*p<0.05 vs. *db*/+; †p<0.05 vs. 6 week.

these mice exhibit altered substrate metabolism, characterized by an over-reliance on FAs for cardiac energy production and low contribution of glucose.[51,52,78] Aasum et al.[51] made the important observation that changes in cardiac metabolism in *db/db* mice preceded the development of cardiac dysfunction (**Fig. 2**) (including increased susceptibility to ischemia-reperfusion), indicating a causal relationship between altered cardiac metabolism and the development of ventricular dysfunction in diabetes.

Later studies demonstrated ventricular dysfunction, not only in *db/db* hearts, but also in hearts from diet-induced obese mice.[51,52,78-80] As mentioned above, these hearts show metabolic shifts towards predominant FA utilization, and the MVO_2-PVA relationships obtained from these hearts were also lifted upward relative to those of normal mouse hearts[53,79] (**Fig. 3A**). These results therefore demonstrate that not only acute elevation in myocardial FA oxidation (as discussed above), but also chronic elevation of FA oxidation, results in decreased cardiac efficiency (i.e. the ratio between MVO_2 and cardiac work). Furthermore, by unloading and chemically arresting hearts, it was shown that the increased oxygen consumption of hearts in diet-induced obese mice was due to increases in both excitation-contraction coupling and basal metabolism (**Fig. 3B**).[53]

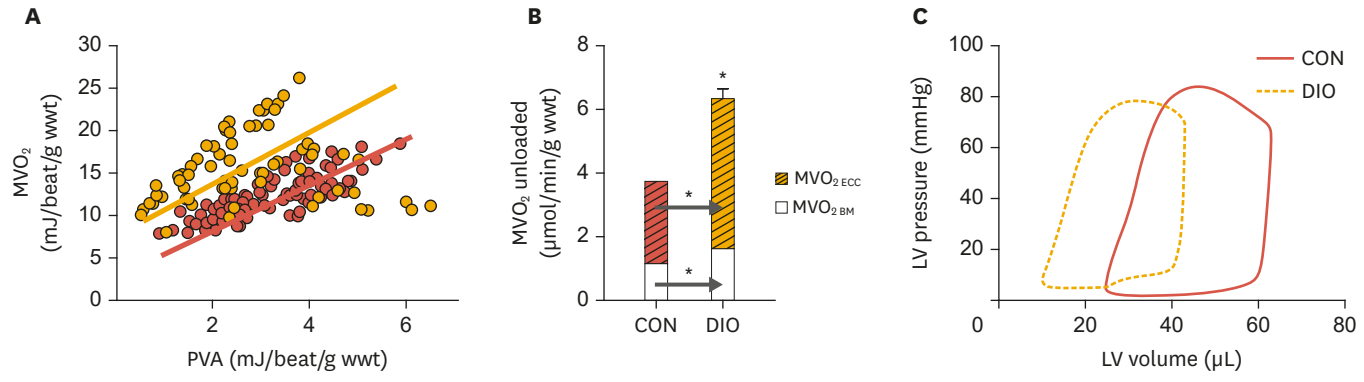

Fig. 3. Increased myocardial oxygen consumption and ventricular dysfunction in DIO mice. (A) Relationship between MVO_2 and total cardiac work (measured as PVA) in isolated perfused hearts from lean CON (red line) and DIO mice (yellow line). (B) The increased oxygen consumption of the DIO hearts is explained by increased oxygen cost for excitation-contraction coupling as well as for basal metabolism. (C) Leftward shift of the pressure-volume loop of DIO heart relative to control, indicating concentric remodeling and ventricular stiffness. Modified from Hafstad et al.[53]
MVO_2, myocardial oxygen consumption; PVA, pressure-volume area; CON, control; DIO, diet-induced obese; LV, left ventricular.
*p<0.05 vs. CON.

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Further examination of ventricular function in hearts from both diabetic and obese mice by pressure-volume analysis clearly revealed diastolic dysfunction, both in hearts from *db*/*db* mice[74] and in hearts from diet-induced obese mice[53] This change in LV function was reflected in a marked leftward shift in the pressure-volume loop (**Fig. 3C**), indicative of the development of concentric remodeling.[79,81,82] In accordance with previous studies on diabetes-induced cardiac remodeling, the hearts exhibited increased fibrosis, impaired metalloproteinase expression, and elevated oxidative stress.[83,84] Park et al.[85] also reported that chronic high-fat feeding and obesity in mice impaired myocardial glucose metabolism, which was associated with ventricular hypertrophy and cardiac dysfunction. The same group reported that diet-induced obesity in mice led to increased macrophage and cytokine levels in the heart, which was associated with significant reductions in AMPK phosphorylation and down-regulation of glucose metabolism.[25]

In summary, the healthy heart is characterized by a high degree of metabolic flexibility, allowing optimal matching of metabolic supply and demand. During conditions of insulin resistance and diabetes, the cardiac muscle is not able to switch effectively from FAs to glucose metabolism in the post-prandial state. As a consequence, the heart becomes metabolically less flexible and ineffective in adapting its fuel preferences to altered energy supply and demand. When relying primarily on FA oxidation for energy production, the heart uses more oxygen for a given workload, compared with a heart oxidizing a mixture of FAs and glucose. The FA-induced elevation in MVO_2 is due to increased oxygen use for non-contractile processes (i.e., basal metabolism and excitation-contraction coupling). The development of both ventricular dysfunction and mechanoenergetic impairments in diabetes/obesity is clearly multifactorial and complex and, in addition to alterations in myocardial substrate utilization, the involvement of Ca^{2+} handling, oxidative stress, mitochondrial dysfunction, and structural remodeling has been proposed.[49,50,65,86-88] Diabetes is also associated with impaired myocardial Ca^{2+} handling, including increased ryanodine receptor 2 Ca^{2+} leak,[88,89] which most likely contributes to the increased oxygen consumption demonstrated herein and in previous studies.[75,79,80,90,91]

TREATMENT STRATEGIES

The obvious solution to prevent adipose tissue inflammation and the accompanying metabolic and cardiovascular complications is to apply strategies for the targeted reduction of this particular fat store in obese individuals. Lifestyle interventions, including changes in diet and physical activity, remain the cornerstone of treatment for obesity and insulin resistance. Both reduced calorie intake and increased calorie expenditure via daily exercise should result in weight loss, but these interventions have not been effective in achieving lasting weight loss. A major part of lost weight is regained within 1 year following the end of treatment, and almost all weight is regained within 5 years.[92,93] The pharmaceutical industry has therefore developed a number of anti-obesogenic medications, including some developed for maintenance of insulin sensitivity. However, several of these agents have been withdrawn from the market due to safety concerns.[93] A new class of anti-diabetic drugs, sodium-glucose cotransporter-2 inhibitors,[94] could hold promise for combatting the obesity epidemic in the future. Although their main effects are to inhibit glucose reabsorption in the renal proximal tubular cells and to reduce blood glucose levels through increased glycosuria, some of these drugs (dapagliflozin and canagliflozin) have been shown to reduce body weight through reductions in fat mass, including both visceral fat and subcutaneous fat.[36,95] Liraglutide

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(Saxenda) is a glucagon-like peptide-1 receptor agonist that was developed for the treatment of type 2 diabetes. It turned out, however, that liraglutide is also an effective treatment for obesity,[96] in part through its actions in the limbic system of the brain,[97] regulating appetite and calorie intake. Pharmacotherapies to prevent obesity will not be further discussed in this review, however, and readers should refer to sources such as the comprehensive review by Van Gaal and Dirinck.[93]

In the final section, we will briefly focus on the use of marine omega-3 FAs in the control of energy homeostasis and their potential role in weight management due to their anti-inflammatory and insulin-sensitizing effects. Long-chain omega-3 polyunsaturated FAs (PUFAs) from fish oil are considered to have beneficial health effects.[98] Thus, treatment of severely obese non-diabetic patients with eicosapentaenoic acid and docosahexaenoic acid was shown to reduce adipose tissue mass and systemic inflammation.[99] A recent meta-analysis of 13 randomized controlled trials, which included over 120,000 participants, confirmed that PUFA supplementation reduces the risk for CHD and CVD, myocardial infarction, and death due to CHD and CVD.[100,101] A systematic review and meta-analysis by Natto et al.[102] also concluded that PUFA consumption may be associated with lower plasma levels of inflammatory biomarkers in patients with diabetes. However, results regarding the effects of PUFAs on glucose metabolism, insulin resistance, and type 2 diabetes are less clear,[103] most likely due to differences in the choice of PUFA preparation, dosage, and intervention.[104] Although the benefits of PUFA intake remain controversial for some diseases and conditions, the anti-inflammatory effects of these compounds are well accepted.[105]

We have previously reported that dietary supplementation with a small amount of oil from the marine crustacean, *Calanus finmarchicus*, reduced both intra-abdominal and hepatic fat deposition, while simultaneously exerting a strong anti-inflammatory action in adipose tissue during high-fat feeding in male C57bl/6J mice.[13] Recently, we also reported[106] that dietary supplementation with *Calanus* oil was able to prevent the obesity-induced decline in myocardial glucose utilization in hearts from high-fat–fed mice. More importantly, post-ischemic recovery of these hearts was significantly better than that of hearts from mice on a non-supplemented high-fat diet, indicating the cardioprotective properties of the *Calanus* oil in obesity. Of note, this effect was achieved with a much lower dose (2%, w/w) than was used in similar experiments in the past.[107] It should be emphasized that the above study included female mice, and in contrast to results obtained with male mice that obesity impaired the recovery of cardiac function after an ischemic insult,[53,78,80] we observed that the post-ischemic recovery of ventricular function in hearts from high-fat—fed female mice was not impaired relative to hearts from mice receiving normal chow. This result confirms previous observations by Edland et al.,[108] who reported that cardioprotection was afforded by long-term feeding of an obesogenic high-fat diet in hearts from female mice. In addition to other possible sex differences, mRNA expression of TNF-α and IL-6 in adipose tissue was hardly detectable in response to high-fat feeding in the female mice, in contrast to previous results with male mice.[12,13] The low inflammatory status could probably be explained by the finding that high-fat feeding induced only a relatively mild degree of adiposity in the female mice, so that the signal for adipokine secretion[17] was missing. In addition, it has been reported that the genes involved in inflammation are more highly up-regulated in males than in females.[109] Still, dietary *Calanus* oil resulted in less deposition of intra-abdominal fat than in untreated high-fat–diet mice. The underlying mechanism is not clear, but increased adipose tissue lipolysis and/or decreased lipogenesis, as well as increased hepatic drainage of FAs from the abdominal fat stores, are possibilities that could be further investigated. Although clinical

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studies are sparse, recent studies in elderly untrained overweight participants[110] suggested that a combination of moderate exercise and intake of oil from *C. finmarchicus* may promote fat loss. It was also shown that wax ester—bound PUFAs from *Calanus* oil were significantly incorporated into the membranes of red blood cells, thereby increasing the omega-3-index.[111]

CONCLUSION

Adipose tissue appears to act as a priming tissue that initiates inflammation in obesity in response to excess energy intake. Thus, obesity-induced dysfunction of visceral and ectopic adipose tissue, including the release of proinflammatory cytokines and FA, is a major contributor to potential pathogenic mechanisms leading to insulin resistance and type 2 diabetes. Preclinical studies have demonstrated that these conditions are associated with a marked shift in myocardial metabolism towards predominant FA utilization for energy production. Over time, this switch in myocardial metabolism leads to a lipotoxic milieu and subsequent metabolic and functional derangements in the heart. Prevention of obesity-related cardiometabolic disease should therefore focus on anti-obesogenic strategies to restore normal adipose tissue metabolism, and understanding the inflammatory responses in adipose tissues of obese individuals is therefore of clear clinical importance.

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